VOLUME XXXIII MAY, 1954 NUMBER 5

The Journal of Clinical Investigation

EDITED FOR THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

Contents

The Effect of Hydrocortisone and of Piromen *In Vitro* on Leukocytes of Patients Receiving ACTH and Cortisone Therapy. GRACE P. KERBY AND JOHN A. BARRETT, JR. 725

A Study of the Renal Circulation, Tubular Function and Morphology, and Urinary Volume and Composition in Dogs following Mercury Poisoning and Transfusion of Human Blood. HADLEY L. CONN, JR., LEE WILDS, AND JOHN HELWIG 732

Studies of Microbial Populations Artificially Localized *In Vivo*. I. Multiplication of Bacteria and Distribution of Drugs in Agar Loci. CHARLES A. WERNER, VERNON KNIGHT, AND WALSH MCDERMOTT 742

Studies of Microbial Populations Artificially Localized *In Vivo*. II. Difference in Antityphoidal Activities of Chloramphenicol and Chlortetracycline. CHARLES A. WERNER AND WALSH MCDERMOTT 753

Studies of Diphtheria Antitoxin in Rheumatic Fever Subjects: Analysis of Reactions to the Schick Test and of Antitoxin Responses following Hyperimmunization with Diphtheria Toxoid. WILLIAM J. KUHNS AND MACLYN MCCARTY 759

The Cardiac Output in Patients with Cirrhosis of the Liver and Tense Ascites with Observations on the Effect of Paracentesis. HENRY J. KOWALSKI, WALTER H. ABELMANN, AND WILLIAM F. MCNEELY 768

Renal Excretion of Electrolytes in Premature Infants during Administration of Sodium Salts of Unreabsorbed Anions. HELEN MCNAMARA AND HENRY L. BARNETT 774

Hepatic Cirrhosis: Effects of Sodium Chloride Administration and Restriction and of Abdominal Paracentesis on Electrolyte and Water Balance. GEORGE J. GABUZDA, JR., HENRY S. TRAEGER, AND CHARLES S. DAVIDSON 780

Genetic Linkage between Ovalocytosis and the Rh Blood Type. RICHARD A. MARSHALL, ROBERT M. BIRD, H. K. BAILEY, AND ESTHER BECKNER 790

Biochemical Study of Muscle in Progressive Muscular Dystrophy. JEAN-CLAUDE DREYFUS, GEORGES SCHAPIRA, AND FANNY SCHAPIRA 794

The Volume and Distribution of Blood in the Human Leg Measured *In Vivo*. I. The Effects of Graded External Pressure. JULIUS LITTER AND J. EDWIN WOOD 798

The Effect of Potassium Chloride on Hyponatremia. JOHN H. LARAGH 807

The Journal of Clinical Investigation

Printed by the Lancaster Press, Inc., Prince and Lemon Sts., Lancaster, Pa. Published by the American Society for Clinical Investigation. Publication Office, 622 West 168th St., New York 32, N. Y.

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Acceptance for mailing at special rate of postage provided for in paragraph (d-2), section 34.40, P.L. & R. of 1948, authorized February 13, 1950

Entered as second-class matter September 16, 1931, at the Post Office at Lancaster, Pa., under the Act of March 3, 1879

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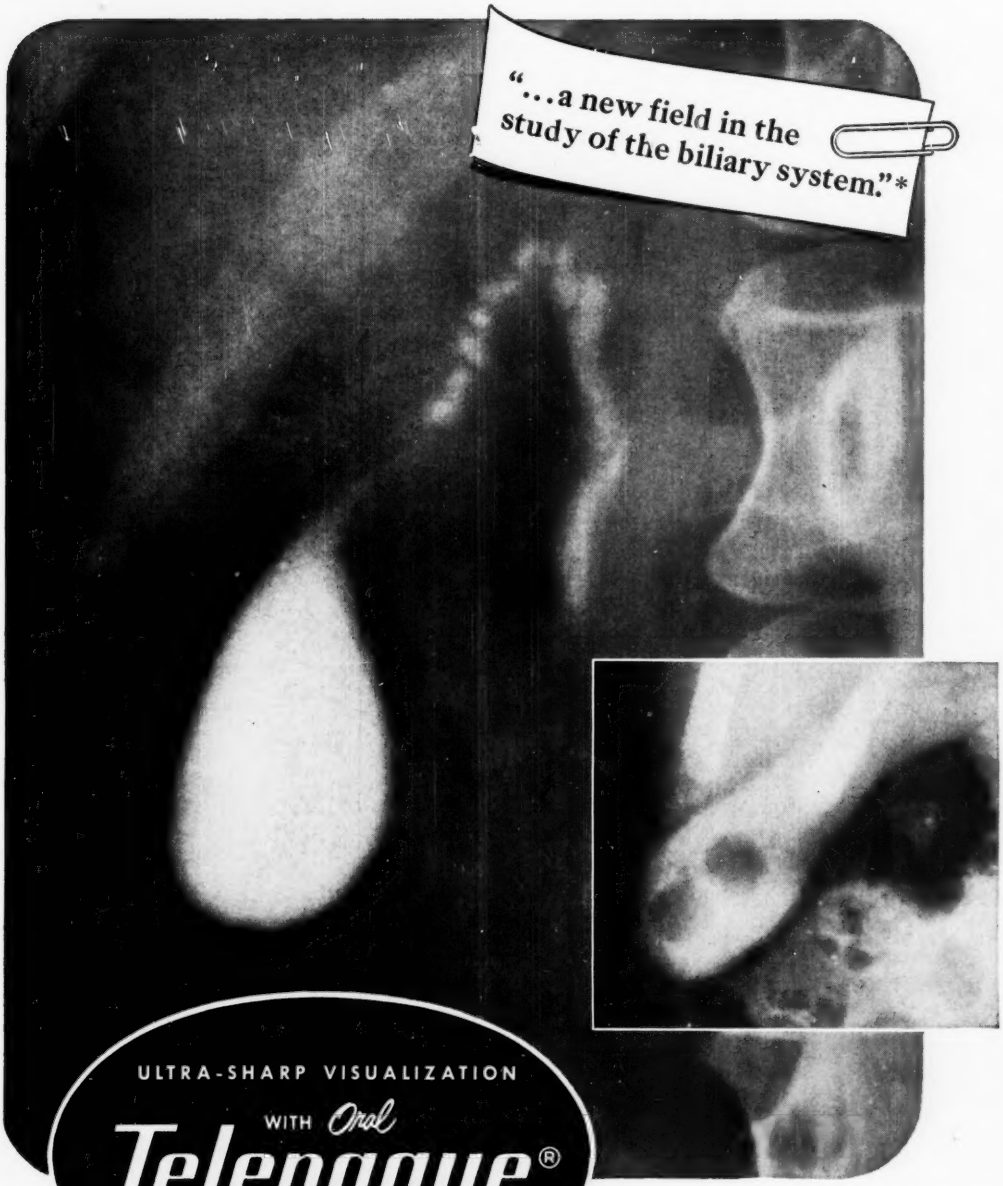

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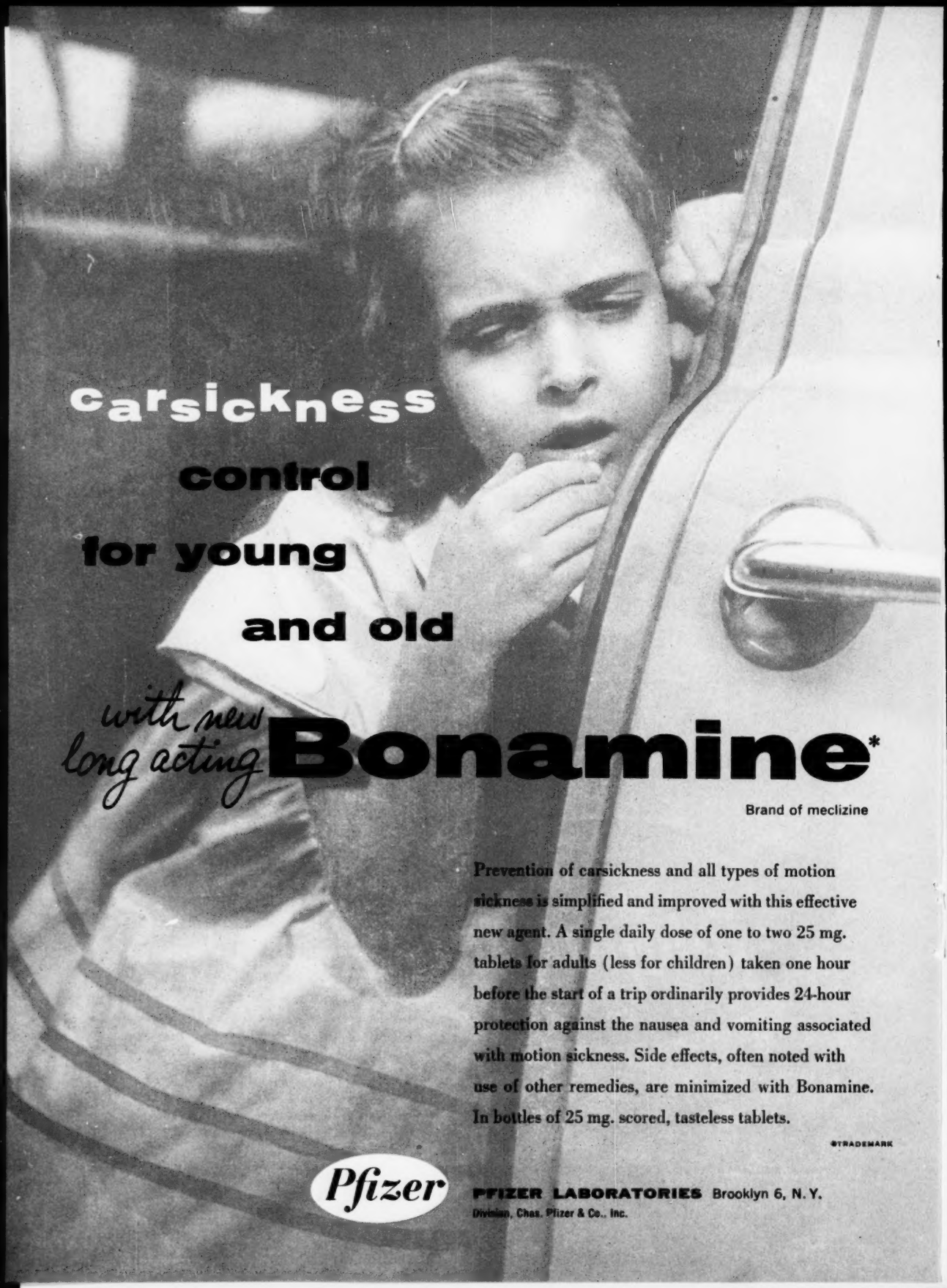
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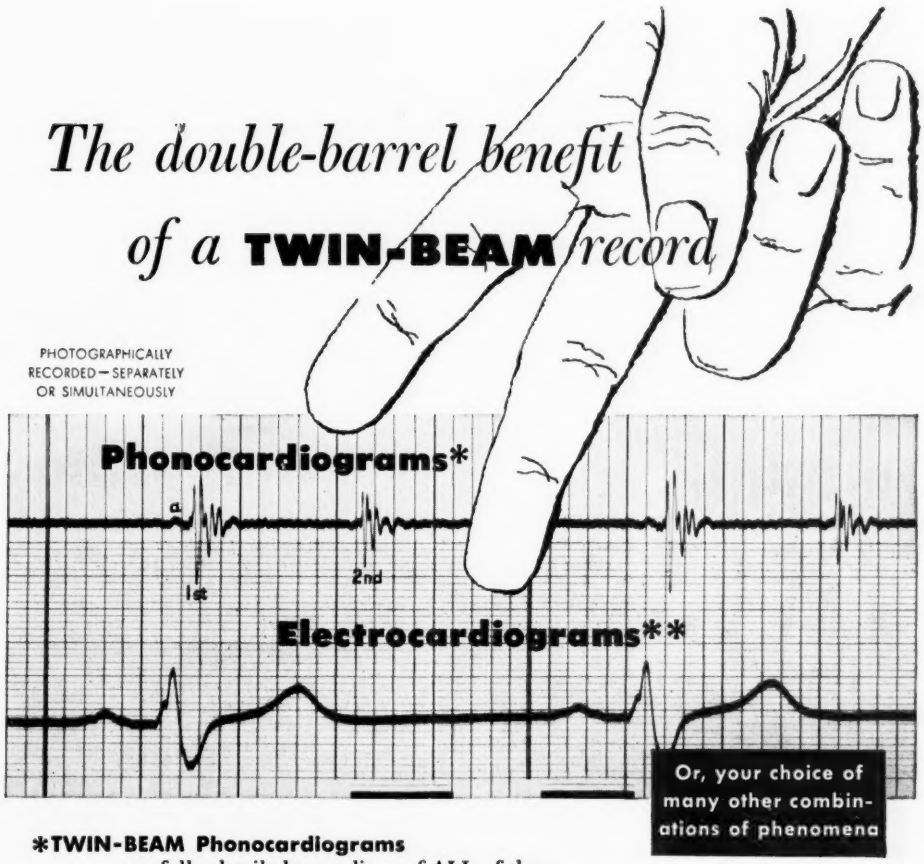

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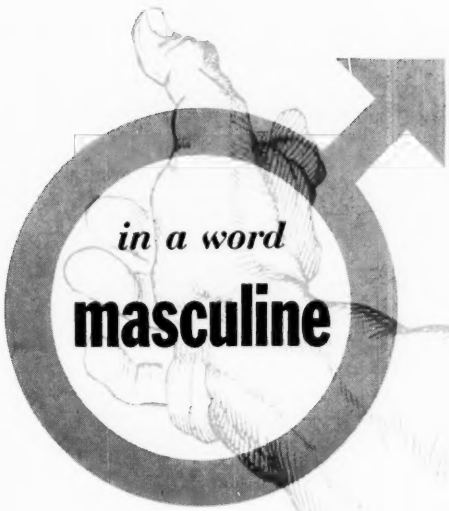

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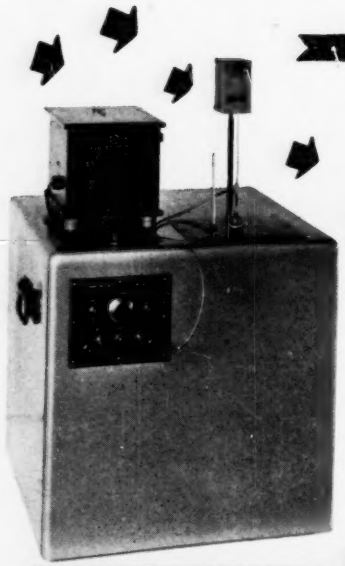

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THE EFFECT OF HYDROCORTISONE AND OF PIROMEN *IN VITRO* ON LEUKOCYTES OF PATIENTS RECEIVING ACTH AND CORTISONE THERAPY [1]

By GRACE P. KERBY AND JOHN A. BARRETT, JR.

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(Submitted for publication October 23, 1953; accepted January 6, 1954)

The relationship of adrenocorticosteroid hormones to the processes of infection, inflammation and repair have been reviewed by several authors, including Duke-Elder and Ashton (1), Rebuck and Mellinger (2), and Thomas (3). Selye (4) has described the overall effect produced in his experiments as an inhibition of development of granulomatous barriers between irritants and tissues. Opinions vary as to how alterations in the response of tissues are induced by the adrenocorticosteroids. Speculation on the basis of data accumulated thus far has dealt particularly with the question of the relative importance of indirect vascular effects and of more direct metabolic effects upon the leukocytes and the connective tissue elements involved in the reaction. Prevention of increased capillary permeability (5), slight increase in arteriolar tone (6), prevention of damage to the endothelium of arterioles and venules (7) and delay of growth of capillary loops (8, 9) have all been observed as vascular effects possibly contributing to the decreased cellular response at the site of injury. There have been numerous attempts made both *in vivo* and *in vitro* to dissociate the direct cellular reaction from vascular and other influences. Shapiro, Taylor, and Taubenhaus (10), for instance, noted the persistence of inhibition of formation of granulation tissue in animals treated with cortisone despite the fact that the site of injury had been denervated and made moderately ischemic. However, Steen (11) was unable to demonstrate any inhibition of fibroblastic growth by cortisone in chick embryonic tissue cultures when the cortisone level approximated the usual human therapeutic range. Gerarde and Jones (12) observed inhibition of fibroblasts in tissue culture only when the embryo from which the tissue was later obtained had been treated *in vivo* with cortisone. The conflicting observations *in vivo* and *in vitro* suggest that cellular alteration may take place *in vivo*, perhaps by indirect means, but that this alteration may persist *in vitro*, once established, at least in embryonic tissues. However, there are other indications (as in the work of Martin, Chaudhuri, Green, and McKinney [13], possibly Huisman [14], and Layton [15]) that alteration of cellular metabolism can be induced by the action of cortisone, entirely *in vitro*.

The present work was undertaken to determine if a direct cellular alteration is demonstrable in man during ACTH or cortisone therapy and if the change persists when the tissue is removed from homeostatic influences to an environment *in vitro*. Leukocytes were studied, because these cells are readily and repeatedly available and they play a role in the general response of the body to injury. As an index to cellular injury, the release of an enzyme (lysozyme) from the cell was quantitated by measuring the amount of lysozyme appearing in the suspending medium under test conditions, and the effect of ACTH and cortisone upon this response of the leukocyte to injury was determined.

METHODS

Subjects. Control patients were individuals not receiving adrenocorticosteroid therapy. They were a heterogeneous group otherwise, ranging from patients with no evidence of organic disease to seriously ill individuals, some of whom were subsequently treated with adrenocorticosteroids. Patients with diabetes mellitus or primary blood dyscrasias affecting leukocytes were excluded from the study.

Test patients were individuals receiving intravenous ACTH or oral cortisone in the course of therapy of various diseases. These included scleroderma, disseminated lupus erythematosus, cranial arteritis, sarcoidosis, optic neuritis, ulcerative colitis, asthma, pemphigus, thrombocytopenic and non-thrombocytopenic purpura, and nephrosis. Although serial observations were made in most

[1] This investigation was supported by research grants from the National Microbiological Institute of the National Institutes of the Public Health Service (E-520), the Duke University Research Council and the Baxter Laboratories, Inc.

TABLE I

Comparison of the in vitro release of lysozyme from leukocytes of untreated patients and patients receiving ACTH or cortisone therapy

Patients	No. of pts.	Per cent (+S.D.) lysis* of lysozyme substrate by leukocytes exposed *in vitro* to: Saline	Piromen	Hydrocortisone	Hydrocortisone + Piromen
A. Controls (no steroid Rx)	26	23.6 ± 9.8	40.4 ± 11.4	16.8 ± 8.0	29.5 ± 11.3
On i.v. ACTH	8	10.1 ± 5.5	18.0 ± 9.2	8.1 ± 4.7	14.0 ± 7.5
Comparison of corrected values t		3.702	5.047	2.729	3.767
Comparison of corrected values p		<0.01	<0.01	<0.01	<0.01
B. Before Rx	8	26.1 ± 6.4	40.3 ± 8.0	19.6 ± 4.9	30.3 ± 6.5
On high-dosage cortisone	8	15.3 ± 4.0	23.6 ± 6.1	13.0 ± 3.5	16.5 ± 5.7
Comparison of corrected values t		4.039	4.709	3.115	4.526
Comparison of corrected values p		<0.01	<0.01	<0.01	<0.01
C. Controls (no steroid Rx)	26	23.6 ± 9.8	40.4 ± 11.4	16.8 ± 8.0	29.5 ± 11.3
On maintenance cortisone	7	20.5 ± 7.1	37.1 ± 10.0	14.8 ± 8.3	26.5 ± 11.7
Comparison of corrected values t		0.788	0.680	0.539	0.605
Comparison of corrected values p		>0.05	>0.05	>0.05	>0.05

* Corrected for plasma residue (see Text, Experimental Procedure).

of the patients, the data presented here are those obtained after five days or more of ACTH (20 units given intravenously over 18 hours) or seven to nine days of high-dosage (300 mg. per day) oral cortisone.[2] Patients on maintenance cortisone had been receiving 50 to 100 mg. of the drug per day for at least 12 days and in most instances for more than one month.

In the high-dosage oral cortisone group, it was possible to obtain at least two baseline observations on each patient prior to therapy, so that in this group the patients served as their own controls. In the ACTH and maintenance cortisone groups, baseline studies were not uniformly possible, and comparisons were made with the control group already described.

Experimental procedure. Dextran-sedimented leukocytes prepared by a method previously described (16) were resuspended in a buffered balanced physiologic salt solution[3] containing 186 mg. per cent of dextrose to a final count of 11,000 leukocytes per cmm. Three and two-tenths ml. of leukocyte suspension were added to an equal volume of (a) the balanced salt solution, or (b) the same, containing 30 micrograms per ml. of either cortisone or hydrocortisone, free alcohol, Merck, giving a final count of 5,500 leukocytes per cmm. and a concentration of 15 micrograms of steroid per ml. The mixtures were incubated in 25 ml. sterile siliconed flasks at 37° C. in a Warburg shaker for three hours,[4] at which time 2.8 ml. of the contents of each flask were added to fresh flasks containing 0.7 ml. of (a) the glucose-fortified balanced salt solution or (b) the same containing PS Piromen,[5] 20 micrograms per ml. (to final concentration, therefore, of 4 micrograms of Piromen per ml.). The flasks were then incubated for an additional two hours, after which lysozyme titres were determined by a method previously described (16). In brief, 1.0 ml. of the test suspension was added to 1.5 ml. of lysozyme substrate,[6] and readings were obtained at zero time and at 20 minutes at 540 mμ in a Coleman Junior Spectrophotometer, the blank containing 1.0 ml. of the test suspension and 1.5 ml. of buffer. Per cent lysis of the substrate was calculated by the formula: $100 - \left(\frac{O.D._{20} \times 100}{O.D._0}\right)$, where $O.D._0$ and $O.D._{20}$ represent the optical densities at zero time and at 20 minutes, respectively. An increased lysozyme titre on *in vitro* exposure of leukocytes to various agents (as compared to the titre observed in a sample of the same leukocyte suspension not so exposed but otherwise identically handled) was interpreted as evidence of at least one type of leukocyte damage.[7] A decreased lysozyme titre on *in vitro* ex-

[2] The cortisone and hydrocortisone preparations used in this study were made available through the courtesy of Dr. Elmer Alpert of Merck and Co., Inc.

[3] Ten ml. stock solution (7.5 per cent NaCl, 0.75 per cent KCl, 0.1 per cent Na_2HPO_4, 0.12 per cent KH_2PO_4, 0.05 per cent K_2HPO_4) and 7 ml. 1 per cent Na_2HPO_4 per 100 ml., aqueous.

[4] The majority of the observations were made using *in vitro* hydrocortisone. When cortisone was used, the initial incubation period was four hours.

[5] A bacterial polysaccharide derived from Pseudomonas aeruginosa and supplied through the courtesy of Dr. L. G. Ginger of Baxter Laboratories, Inc.

[6] A dried preparation of M. lysodeikticus, obtained commercially as Bacto Lysozyme Substrate and prepared for use as previously described (16).

[7] Under conditions known to produce cellular injury, *e.g.*, a step-wise decrease in salt concentration of the suspending medium, there occurs a step-wise increase in the amount of lysozyme appearing extracellularly for titration. Similarly, the exposure of human leukocytes to an antiserum to human leukocytes or to various damaging bacterial derivatives results in the release of increased amounts of lysozyme into the suspending medium where it can be measured. On the basis of these observations

TABLE II

Comparison of the in vitro lysozyme activity of leukocyte lysates and plasma of untreated patients and patients receiving ACTH or cortisone therapy *

Patients	No. of pts.	Leukocyte lysate, 1:10 in water†	Plasma, 1:10 in saline
A. Controls (no steroid Rx)	26	41.6 ± 11.6	20.2 ± 10.0
On i.v. ACTH	8	27.2 ± 13.5	12.0 ± 3.8
Comparison of data t		2.437	1.943
Comparison of data p		<0.05	>0.05
B. Before Rx	8	39.6 ± 10.3	27.2 ± 15.2
On high-dosage cortisone	8	35.9 ± 11.6	16.8 ± 8.0
Comparison of data t		0.675	1.713
Comparison of data p		>0.05	>0.05
C. Controls (no steroid Rx)	26	41.6 ± 11.6	20.2 ± 10.0
On maintenance cortisone	7	34.6 ± 16.4	17.4 ± 6.9
Comparison of data t		0.689	0.719
Comparison of data p		>0.05	>0.05

* Data represent per cent (±S.D.) lysis of lysozyme substrate by the test material.
† Corrected for plasma residue (see Text, Experimental Procedure).

posure of leukocytes to adrenocorticosteroids was interpreted as evidence of protection of the cells from injury.

Leukocyte lysate titres were determined as described previously (18) using a 1:10 dilution of the lysate for titration. The titres represent the maximal amount of lysozyme which is released from leukocytes on exposure of the cells to distilled water.

Plasma titres of lysozymes were determined, using 1 ml. of a 1:10 dilution of plasma plus 1.5 ml. of lysozyme substrate. The lysozyme activity of plasma is a factor of importance in the present study because of the trace of plasma residue left on the leukocytes in the method used. The method avoids all excess handling of the leukocytes, either for purposes of washing them or for measuring plasma residues. Where comparisons were made between *in vitro* treated and untreated leukocytes, as in Table II, no error was introduced, since all of the leukocytes were from the same suspension with the same plasma contamination. However, where comparisons were drawn between leukocytes obtained from the patient before and after treatment with steroids, an error was introduced which tends to increase the difference between the observations before and after therapy. This occurred because, with the marked rise in leukocyte count which appeared during therapy, final dilution of any plasma residue became proportionally high as counts were adjusted to 11,000 per cmm. Also, plasma titres of lysozyme tended to decrease with therapy. It is apparent, therefore, that a correction must be made for plasma lysozyme activity [8] before any valid comparison can be made. For all comparisons before and after treatment of the patient (Tables I and II, Figures 1 and 2), therefore, only those patients were included on whom complete data were at hand concerning individual plasma lysozyme titres and final volume of leukocytes after dilution to 11,000 per cmm. With these data and the mean plasma residue,[8] correction through the possible range of lysozyme activity due to plasma contamination could be made for both leukocytes and leukocyte lysates.

In Table I-A and -B, the activity of the mean plasma residue + 2 S.D. (or 0.1734 ml.) was deducted from the control or baseline observations, whereas the mean plasma residue − 2 S.D. (or zero) was deducted from the observations after therapy. Thus, the difference in the levels before and after therapy was minimized to the greatest extent that the plasma artefact of the experimental method could have influenced the results, and the significance of the remaining, considerably-lessened difference was then tested. This was not possible in Table I-C, because there was insufficient difference between control and treated patients to permit such an approach to the data. Therefore, the maximum possible deduction for plasma activity was made from both sets of observations in this instance before testing the significance of the difference between observations.[9] The same procedure was carried out in preparing the data for Figures 1 and 2.

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which are reported in detail elsewhere (16–18), an increased titre of lysozyme in the medium in which the cells are suspended is interpreted as one index of cellular injury.

[8] A control experiment was run, in which the plasma-platelet-dextran supernate was removed by suction from ten identical tubes of centrifuged leukocytes, using the same technique as was used throughout the experiments presented here. The plasma residue was then carefully measured, and the mean residue volume was found to be 0.083 ml. ± 0.0452 (S.D.).

[9] For example, in Table I-A, column "saline," the uncorrected lysozyme levels were 31.2 ± 10.5 and 10.1 ± 5.5 for leukocytes from untreated and treated patients, respectively. Following maximum correction for possible plasma residue contamination, the lysozyme levels which were finally used for comparison were 23.6 ± 9.8 and 10.1 ± 5.5, respectively. In Table I-C, column "saline," the uncorrected levels were 31.2 ± 10.5 and 29.6 ± 10.8,

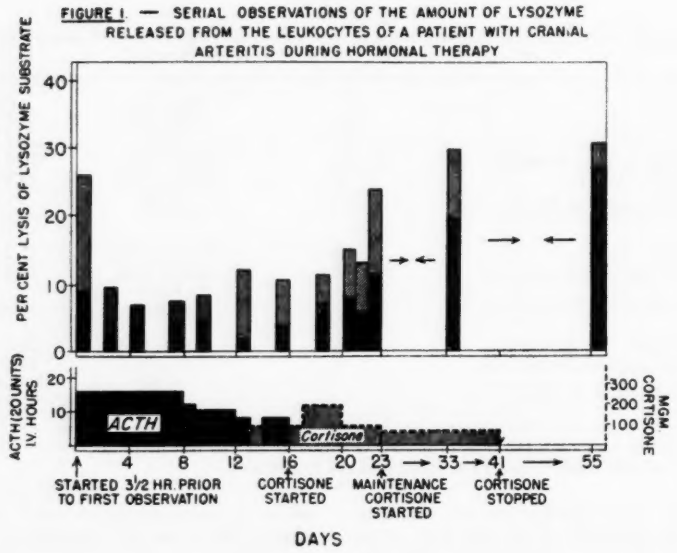

FIGURE 1

The black bar represents the per cent lysis of lysozyme substrate by leukocytes subjected to the minimal trauma of handling. The cross-hatched bar demonstrates the superimposed cellular injury (expressed as increased release of enzyme) when the same leukocyte suspension was exposed to Piromen *in vitro*. All observations have been corrected for plasma contamination.

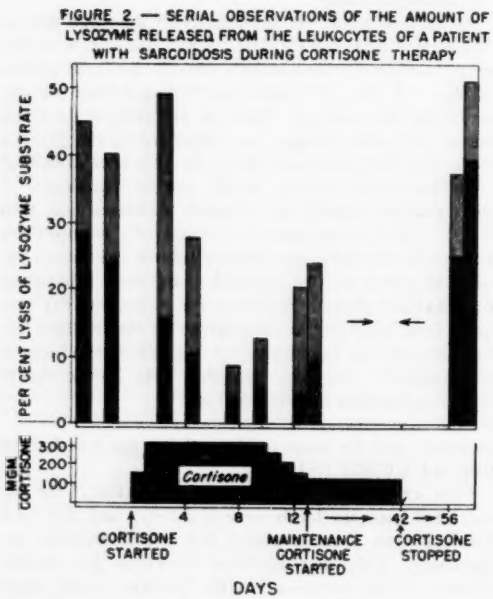

FIGURE 2

Representations by the black and cross-hatched bars are as for Figure 1.

RESULTS

The results are summarized in Tables I to III.[10] Serial observations were obtained on most of the patients, in same instances prior to therapy, through intravenous ACTH or high-dosage oral cortisone to maintenance cortisone or no therapy. This is graphically illustrated in Figures 1 and 2 for two patients treated with intravenous ACTH and oral cortisone, respectively. All patients showed the same trend, although not all to the same degree as illustrated by these two patients, as is evident from the mean values recorded in the tables.

respectively; after correction they were 23.6 ± 9.8 and 20.5 ± 7.1, respectively.

[10] Five patients were given a single four-hour intravenous infusion of 100 mg. of hydrocortisone (free alcohol) Merck in 5 per cent dextrose in water. Observations were made prior to and after the infusion (immediately after, at four hours and at 24 hours). A trend toward a decrease in susceptibility of the patient's leukocytes to injury was observed, disappearing after 24 hours. However, this did not occur in all of the patients, and the overall significance of the trend could not be adequately evaluated in the small series. For this reason, the data are not included in the present report.

TABLE III

Effect of exposure of leukocytes from steroid-treated and untreated humans to in vitro hydrocortisone and Piromen

	Mean difference (±S.D.) in lysozyme activity between a leukocyte suspension and the same suspension exposed *in vitro* as indicated by†				
	*(1)	(2)	(3)	(4a)	(4b)
No. of Pts.	39	11	8	8	8
(A)†	+16.8 ± 7.6	+17.0 ± 5.3	+7.9 ± 4.3	+14.2 ± 2.4	+8.3 ± 3.4
t	13.79	10.70	5.172	17.04	6.918
p	<0.01	<0.01	<0.01	<0.01	<0.01
(B)†	−6.2 ± 4.9	−3.9 ± 3.6	−1.8 ± 1.7	−6.5 ± 2.5	−2.4 ± 2.5
t	7.982	3.407	2.887	7.303	2.685
p	<0.01	<0.01	<0.05	<0.01	<0.05
(C)†	+6.2 ± 6.7	+6.7 ± 5.1	+3.8 ± 3.3	+4.2 ± 3.8	+1.2 ± 3.7
t	5.771	4.337	3.250	3.102	0.887
p	<0.01	<0.01	<0.02	<0.02	>0.05

* (1) Untreated patients.
(2) Patients receiving maintenance oral cortisone.
(3) Patients receiving i.v. ACTH.
(4) Patients receiving large dosage oral cortisone.
(a) Before therapy.
(b) After 7–9 days of therapy.

† A. Damaging effect of *in vitro* exposure of leukocytes to PS Piromen.
B. Protective effect of *in vitro* exposure of leukocytes to hydrocortisone.
C. Combined effect of *in vitro* exposure of leukocytes to hydrocortisone and Piromen.

DISCUSSION

The studies presented here indicate that the leukocytes of patients undergoing treatment with ACTH or cortisone are significantly altered in their response to at least two forms of injury (*i.e.*, mechanical trauma and exposure to a bacterial polysaccharide derived from *Ps. aeruginosa*). This alteration persists and is demonstrable when the leukocyte is removed from the host and isolated from the homeostatic influences which might be contributing to any observed alteration. Lessened susceptibility to injury as indicated by lysozyme activity does not coincide with the disappearance of eosinophils but usually makes its appearance later, sometimes becoming maximal after as long as seven to nine days of treatment. It occurs only with high-dosage hormonal therapy over an adequate period of time. Leukocytes revert to normal when hormonal therapy is discontinued or when maintenance dosage is instituted. When such patients are again treated with large doses of hormones (because of clinical relapse), the leukocytes again tend to show resistance to injury, suggesting that the reversion to normal observed during maintenance therapy is not part of a homeostatic adjustment by the cell but rather a question of size of dose of adrenocorticosteroids.

The same lessened susceptibility to injury can be induced in leukocytes of patients who have received no hormonal therapy by exposing the cells to cortisone or hydrocortisone *in vitro*. When the leukocytes are obtained from treated patients, such a protective effect *in vitro* can also be demonstrated, but it is less in degree than in the untreated group. This suggests that there may be a limit to the degree of protection which can be bestowed and that in cells partially altered in this regard *in vivo*, the additional protection possible by *in vitro* measures is limited.

Since the method used here for detection of injury to the cell depends on the measurement of a substance (lysozyme) released from the cell into the surrounding medium, it becomes pertinent to inquire whether the substance measured might be absolutely decreased in the cell while the host was being treated with ACTH or cortisone. For this reason, the lysozyme activity of water-lysed leukocytes was measured at the same time that the intact leukocytes were studied. There was no evidence of decreased lysozyme content in the leukocytes of patients treated with cortisone, either in maintenance or high dosage. However, in the leukocytes of patients receiving intravenous ACTH, there was a decrease in demonstrable lysozyme, and the magnitude of the decrease was of possible significance, statistically.

It seems likely that the observed effects reflect an alteration in the metabolism of the leukocyte which persists during the life of the cell after it has been removed from the host. This concept is supported by the studies of Martin, Chaudhuri, Green, and McKinney (13), Huisman (14), and Layton (15), who have described specific alterations in metabolic processes of tissues exposed *in vitro* to cortisone. The present studies suggest that some metabolic changes do not depend on influences other than the hormone itself, because essentially the same effect observed in leukocytes from patients receiving ACTH or cortisone therapy could be induced in normal leukocytes by exposure of the cells to cortisone or hydrocortisone *in vitro*. Indeed, the observed changes may be opposed by other influences *in vivo*, since it is evident that the hormonal effect on leukocytes was achieved much more rapidly *in vitro* than *in vivo*. On the other hand, the effect *in vivo*, once fully developed, exceeded that obtained *in vitro*,[11] despite the fact that the hormonal level *in vitro* was undoubtedly higher than that in the blood of the treated patient.

It is becoming increasingly evident (19) that the intact organism makes adaptations at various levels during the course of adrenocorticosteroid hormonal therapy and that a given response *in vivo* cannot be interpreted as necessarily due to a direct effect of the hormone administered. The response may be due, wholly or in part, to inhibitory or synergistic substances produced by cells in response to the hormone. In the present study, a direct hormonal effect on the cells was demonstrated when they were exposed to the hormone *in vitro*. However, this effect occurred within five hours *in vitro*, whereas a number of days was required for the development of a similar effect when the hormone was administered *in vivo*. Further investigations will be necessary to determine why these two responses differ.

SUMMARY

1. By a method which measures release of lysozyme from cells (an index of cellular injury), the effect of adrenocorticosteroids on the susceptibility of human leukocytes to injury has been investigated.

2. Leukocytes from patients receiving intravenous ACTH or oral cortisone in high dosage released significantly less lysozyme on exposure to injury by mechanical trauma or by a toxic bacterial derivative than did leukocytes from untreated patients. The maximum protective effect of the hormones did not correlate with disappearance of eosinophils and usually required several days for development.

3. Leukocytes from patients on smaller doses of cortisone did not differ from controls in their susceptibility to injury as measured by the release of lysozyme from the cells.

4. Lessened susceptibility to injury could be induced in leukocytes from untreated patients and patients on maintenance cortisone therapy by exposure of the cells *in vitro* to cortisone or hydrocortisone. The effect was apparent in five to six hours, in contrast to the period of several days necessary for development of a similar effect *in vivo*.

5. Lysates of leukocytes of patients on high-dosage or maintenance cortisone did not differ from controls in their lysozyme content. Lysates of leukocytes of patients receiving intravenous ACTH showed less lysozyme activity than did controls.

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A STUDY OF THE RENAL CIRCULATION, TUBULAR FUNCTION AND MORPHOLOGY, AND URINARY VOLUME AND COMPOSITION IN DOGS FOLLOWING MERCURY POISONING AND TRANSFUSION OF HUMAN BLOOD[1]

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(Submitted for publication August 3, 1953; accepted January 12, 1954)

Many recent publications incriminate some alteration in the renal circulatory bed as a primary factor in the production and/or maintenance of most states of acute renal insufficiency (1–13). On the one hand, measurements of renal blood flow made by use of the Fick principle either at the onset of the injury or subsequent to an ensuing anuric or oliguric period have shown a significant renal ischemia associated with surgical and hemorrhagic shock, abortion, incompatible blood transfusions, heavy metal poisoning, hemoglobin infusions, and carbon tetrachloride poisoning (1–11). The accuracy of some of these measurements is open to question because of the almost negligible renal extraction of the test substance involved and the difficulties of urine collection during severe oliguria. However, this body of data has been interpreted as indicating that an initial renal ischemia is primarily responsible for the development of renal insufficiency and ultimately anuria produced by all insults not directly nephrotoxic; and that renal ischemia probably is present throughout the period of anuria whatever the initial cause. On the other hand, Trueta Raspall (12), using the results of rabbit experiments, has proposed that the important vascular abnormality is an intrarenal "juxtamedullary shunting" of blood presumably with a normal over-all flow. The continuation of this "shunting" throughout the period of renal inadequacy is implied.

Since the "shunt" hypothesis has been widely challenged and the other not yet entirely documented, consideration of these concepts raises certain related questions: 1) Do intrarenal shunts in the intact subject ever play any part in the development or continuation of renal insufficiency states? 2) Does an initial renal ischemia persist to become a major contributor to the continuation of renal insufficiency or to the commonly associated anuria? With the development of a gas diffusion method for measuring renal blood flow which does not require urine for analysis (14) and with the application of newer arteriographic techniques, more direct and more satisfactory answers can now be given to these questions.

In the absence of readily available clinical material, two abnormal conditions which might simulate those leading to acute anuria in humans were created in dogs: bichloride of mercury poisoning and an incompatible blood transfusion reaction. Then at the time of anticipated maximal urinary suppression, the renal circulatory behavior and tubular function were studied by correlating measurements of renal blood flow, intrarenal distribution of flow, renal para-amino-hippurate (PAH) extraction, and renal arteriovenous oxygen differences with urinary and *post mortem* renal morphological changes.

METHODS

The two methods of producing renal or urinary tract abnormalities were: administration of human blood by transfusion, and mercuric chloride poisoning. To simulate human incompatible blood transfusion reaction, the administration of human blood rather than dog blood was resorted to. This was done despite the heterologous nature of the infusion because homologous transfusions in the dog given without regard to blood types have never been noted to cause renal damage. Mercuric chloride was chosen in light of its known ability to cause human anuria. The dosages to be given were decided as follows: the volume of human blood used was that calculated as being equivalent to transfusing 500 ml. of whole blood into an average adult human, and the amount of mercury given

[1] This work was supported in part by the National Heart Institute of the U. S. Public Health Service (H 405) and in part by a University of Pennsylvania Faculty Research Grant.

[2] Research Fellow of the American Heart Association during the tenure of this study.

was that found to cause the greatest renal damage without consistently killing or excessively "shocking" the dog before the studies could be done.

In all, 21 dogs were used. Ten were given 7.5 ml. per Kg. of human blood intravenously 48 to 72 hours previous to the study, while two were given the same dose two and one-half weeks previous to the study and the dose repeated three days previous to the study. Blood of all types secured from a hospital blood bank was used 10 to 15 days after its donation. Control studies were done on all dogs given human blood. Eight dogs were given 15 mg. per Kg. and one, 30 mg. per Kg. of mercuric chloride by the intramuscular route.

In preparation for the renal studies, all animals were lightly anesthetized by an intravenous injection of equal parts of Pentobarbital Sodium and Dial-Urethane, 0.3 to 0.4 ml. per Kg. A No. 8, curved tip, woven venous catheter was then passed under fluoroscopic guidance from the external jugular vein deep into the renal vein (usually the left), so that the tip lay within the shadow of the renal parenchyma. The left renal vein was used because of previous anatomical studies in dogs which showed the segment of renal vein from the renal parenchyma to the spermatic vein on the left to be consistently longer than the entirety of the right renal vein. Thus, the possibility was minimized of including in the sample, blood other than renal venous blood.

Renal venous blood was then collected through a manifold system (15). Arterial blood was obtained *via* a plastic catheter passed through the femoral artery into the aorta, the tip lying superior to the renal artery. This catheter was also connected to a manifold system.

Renal blood flow was measured by the gas diffusion method utilizing nitrous oxide. This principle and this specific method have both been described elsewhere in detail (14, 15). Flow is expressed in ml. per 100 Gm. of renal tissue per minute.

Intrarenal distribution of flow was determined by injecting (in approximately one second) 20 ml. of 70 per cent Iodopyracet into the arterial catheter and taking seven successive radiographs of the kidneys usually 1, 2, 3, 4, 10, 20, and 30 seconds after the injection.

Mean arterial blood pressure was measured by a mercury manometer connected with the arterial catheter-manifold system. Renal resistance was calculated in mm. Hg per ml. per 100 Gm. per min. from the renal flow and the mean arterial pressure. No correction for venous pressure was employed.

Simultaneous arterial and venous samples for oxygen content were drawn into oiled, heparinized 10 ml. syringes and were analyzed manometrically after the method of Van Slyke and Neill (16). The results were corrected for renal arteriovenous hematocrit differences, the largest correction being 3 per cent of the oxygen content. Oxygen consumption was calculated in ml. per 100 Gm. of renal tissue per minute. The hematocrits were determined by centrifugation of samples placed in Wintrobe tubes and spun for 30 minutes at 4000 rpm. in a General Electric size 2 centrifuge.

PAH measurements were done on whole blood. The reason for the use of whole blood determinations, as well as the details of the method involved with the following exception, have been described elsewhere (17). Trichloracetic acid (3 per cent in final solution) was used for protein precipitation in place of cadmium sulfate, since its use resulted in more consistent and essentially complete PAH recoveries. As compared with 85 to 90 per cent renal plasma PAH extraction (E_{PAH}), whole blood extractions were found to be somewhat lower (74 ± 8 per cent), presumably due to slower diffusion of PAH from the red cells.

Blood urea nitrogen was determined in one animal. The amount of urine, if present, was measured, specific gravity determined, and the urine was examined microscopically and tested for albumin.

After the animals were sacrificed, the kidneys were weighed and the weight expressed in Gm. per Kg. of dog weight. The kidneys were then examined both grossly and microscopically.

RESULTS

I. *Human blood* (Table I)

During the initial administration of blood at a rate of approximately 3 ml. per minute, a fall in blood pressure invariably occurred after 10 to 35 ml. This hypotension varied markedly in degree and duration. In animals being given a first infusion, mean systemic pressure falls of 40 to 100 mm. of mercury were observed for periods from 15 minutes to an hour. This phenomenon could be repeated once or twice, but thereafter, the systemic pressure stabilized or tended to rise when the infusion was continued. In the two dogs given a second infusion, the fall in pressure was 60 to 90 mm. of mercury and this was sustained for three hours in one, and over five hours in the other. Unfortunately, renal hemodynamics were not evaluated at this time.

Three of the dogs, including the one which had the longest period of hypotension after a second blood infusion, died before the subacute renal circulatory changes could be determined. Six which were given only a first infusion of blood, showed no evidence of renal abnormality when studied by the techniques described herein at the prescribed 48 to 72-hour period. A complete recapitulation of the data on these animals is therefore excluded from the present report, leaving only the results in the three in which a definite renal effect was demonstrated. These three animals were designated

TABLE I

The effects of the intravenous administration of human blood on the renal circulation, tubular function, urinary volume and composition, and renal morphology of the dog

Experimental state	Urine	*Post mortem* kidneys (5.8–9.0 Gm./Kg.)*	Renal arteriography	H'crit	Mean arterial blood pressure (100–140)	Renal blood flow (290–400)†	Renal resistance (0.25–0.48)‡	Oxygen consumption (5–9)§	Whole blood PAH extraction (66–82%)‖
					mm. Hg	*ml./100 Gm./min.*	*mm./ml./100 Gm./min.*	*cc./100 Gm./min.*	*%*
Control *A*	Normal 1 ml./min.	—	—	—	115	323	0.35	6.6	86
A 7½ ml./Kg. A+ blood 48 hrs. before study	Sp. gr. 1.008 0.25 ml./min. Proteinuria 1+	Vessels hyperemic, slightly pale, not swollen, 7.3 Gm./Kg. "transfusion nephrosis"	Normal	42	110	305	0.36	5.9	57
Control *B*	Normal approx. 1 ml./min.	—	—	—	135	380	0.36	7.2	78
B 7½ ml./Kg. AB+ blood 72 hrs. before study	Sp. gr. 1.010 0.2 ml./min. Proteinuria 1+	Pale, slightly edematous 9.0 Gm./Kg. "transfusion nephrosis"	Slightly prolonged dye shadow. Enlarged kidneys	49	135	295	0.46	5.6	3
Control *C*	Normal approx. 1 ml./min.	—	—	45	125	380	0.33	6.5	75
C 7½ ml./Kg. O+ blood 2½ weeks and 72 hrs. before study BUN—40	Anuria	Pale, edematous "transfusion nephrosis"	Prolonged dye shadow. Enlarged kidneys	43	110	150	0.73	4.8	0

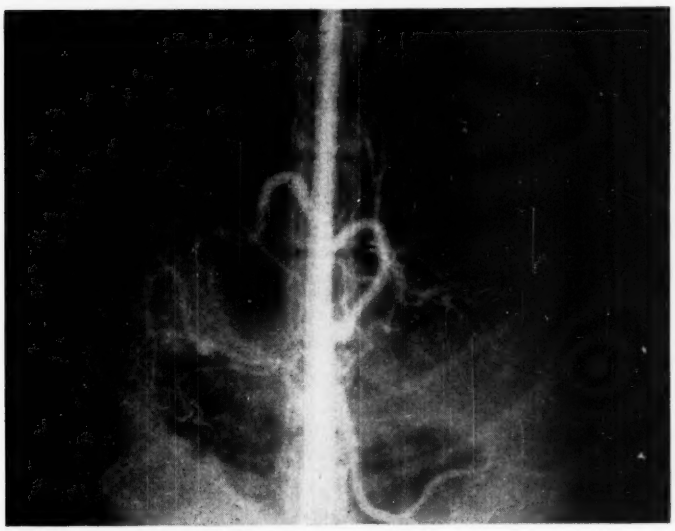
* Normal limits in 15 animals.
† Normal limits in 11 animals.
‡ Calculated from normal limits for B.P. and R.B.F.
§ Normal limits in 11 animals.
‖ 74% ± 8% (2 S.D.) in 12 animals.

as IA, IB, and IC; the latter being the surviving dog given a second blood transfusion. The results were as follows:

Urine. Dogs IA and IB were passing 0.25 ml. and 0.2 ml. of urine per minute, respectively. The urinary specific gravity was 1.008 to 1.010 with slight proteinuria. Dog IC was anuric.

Circulatory bed. In dogs IA and IB, renal blood flow and renal resistance were within normal limits, the flow being slight (5 and 22 per cent), but not significantly less than the control values. Dog IC had a renal blood flow of approximately 40 per cent of normal with an elevated renal vascular resistance. The intrarenal distribution of flow was normal in all, although the prolonged parenchymal dye shadow suggested reduced total flow. Compare the renal arteriogram from a normal dog (Figure 1) with that of dog IC (Figure 2).

Renal function. Renal whole blood PAH extraction (E_{PAH}) fell from 86 to 57 per cent in dog IA, 78 to 3 per cent in dog IB, and 73 to 0 per cent in dog IC. The oxygen consumption remained within normal or borderline normal limits in all three animals. However, the results represented a decrease from the control values in each case. The blood urea nitrogen in dog IC was elevated



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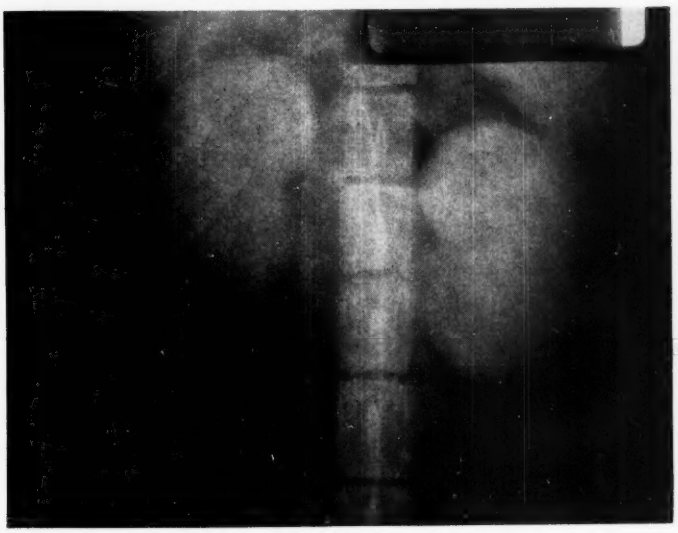


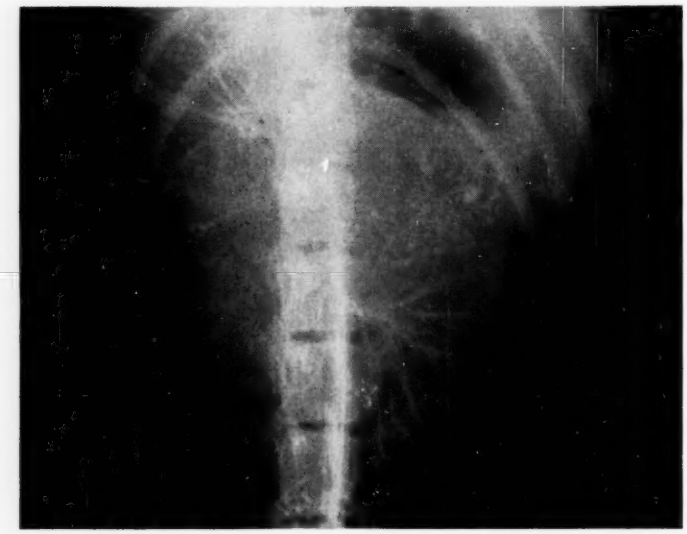
B

FIG. 1. RENAL ARTERIOGRAM OF A NORMAL DOG

Following injection of 20 ml. of 70 per cent Iodopyracet into the aorta through a polyethylene catheter (see Methods), the main renal arterial tree is filled with dye at two seconds (A) and the cortex is diffusely filled with dye at eight seconds (B).

to 40 mg. per cent. Unfortunately, this measurement was not made in the other two animals.

Post mortem examination. The tubular degeneration and necrosis associated with hemoglobin casts in the tubular lumina which have been described as characteristic of "mismatched transfusion nephrosis" were present to a moderate degree in dog IA and in large degree in dogs IB



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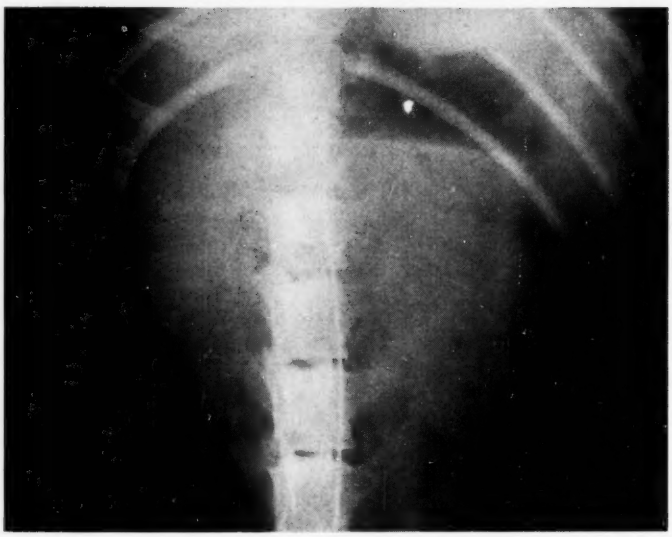

B

FIG. 2. RENAL ARTERIOGRAM OF DOG IC SEVENTY-TWO HOURS AFTER THE ADMINISTRATION OF HUMAN BLOOD

The renal vessels are normally visualized at two seconds and the dye appears equally distributed throughout the cortex at ten seconds. The right kidney, partially obscured by the rib cage, is reproduced poorly in the photograph. The left appears somewhat enlarged.

and IC. The picture seemed to represent a conglomeration of patchy changes rather than involvement of any one anatomical part of the nephron. Degenerative swelling, renal edema, and inflammatory reaction were much more in evidence in dog IC.

II. *Mercury bichloride poisoning* (Table II).

Nine animals were given mercury bichloride; five died from the extrarenal effects before studies were made. The surviving four all showed to a varying extent: dehydration, weakness, lethargy, and bloody diarrhea. The following renal data were obtained in these animals:

Urine. Dogs IIA and IID were passing relatively normal amounts of urine (0.5 to 1.0 ml. per min.) 60 and 54 hours, respectively, after the mercurial injection. Dog IIC was anuric 72 hours after the injection. Dog IIB, while showing little decrease in urinary volume after 48 hours, had become severely oliguric after 12 hours. All dogs passing urine had hyposthenuria with albumin and casts.

Circulatory bed. The renal blood flow was normal in the two dogs showing little or no urinary

TABLE II

The effects of intramuscularly injected mercuric chloride on the renal circulation, tubular function, urinary volume and composition, and renal morphology of the dog

Experimental state	Urine	*Post mortem* kidneys (5.8–9.0 Gm./Kg.)*	Renal arteriography	H'crit	Mean arterial blood pressure (100–140)	Renal blood flow (290–400)†	Renal resistance (0.25–0.48)‡	Oxygen consumption (5–9)§	Whole blood PAH extraction (66–82%)‖
					mm. Hg	*ml./100 Gm./min.*	*mm./ml./100 Gm./min.*	*cc./100 Gm./min.*	*%*
A									
15 mg./Kg. (IM)—$HgCl_2$ 60 hrs. before study	Sp. gr. 1.014 Oliguria minimal 0.4 ml./min.	Grossly normal No microscopic 7.1 Gm./Kg.	—	—	125	355	0.37	—	—
B1									
30 mg./Kg. (IM)—$HgCl_2$ 48 hrs. before study	Sp. gr. 1.011 ++ albumin and casts	—	—	44	115	265	0.43	7.1	57
B2									
Same dog at 120 hrs.	Extreme oliguria (occ. drop) ++++ albumin and casts	Gross-slight swelling & pallor Micro-mod. tubular deg. & necrosis 8.4 Gm./Kg.	Normal distribution Prolonged cortical dye shadow	65	120	170	0.70	3.4	15
C									
15 mg./Kg. (IM)—$HgCl_2$ 72 hrs. before study	Anuric	Grossly normal Micro-marked tubular destruction deg. and necrosis 7.3 Gm./Kg.	Normal	36	73	155	0.47	1.0	5
D									
15 mg./Kg. (IM)—$HgCl_2$ 54 hrs. before study	Sp. gr. 1.007 0.5 ml./min. ++++ albumin and casts	Grossly normal 6.2 Gm./Kg. Micro-similar to Dog C	—	51	118	400	0.30	2.0	4

* Normal limits in 15 animals.
† Normal limits in 11 animals.
‡ Calculated from normal limits for B.P. and R.B.F.
§ Normal limits in 11 animals.
‖ 74% ± 8% (2 S.D.) in 12 animals.

volume change and was only slightly subnormal in dog IIB when first studied. However, at the time when urinary volume was essentially *nil* in dog IIB and in dog IIC, renal blood flow was only approximately 40 to 50 per cent of normal. Intrarenal distribution of blood flow, as determined by arteriography, was normal in all cases studied. The calculated renal resistance was within top normal limits in all studies except in the case of the second study on dog IIB. Here with a blood flow approximately 50 per cent normal (170 ml. per 100 Gm. per min.) and an associated mean pressure of 120 mm. Hg, the calculated mean resistance was 50 per cent above normal. The hematocrit, however, had increased from 44 to 65. Only anuric dog IIC had a mean pressure at shock levels 73 mm. Hg.

Tubular function. Both oxygen consumption and PAH extraction were markedly reduced in the three dogs so studied except the first study on IIB where clearly abnormal values were not demonstrated. This reduction occurred in roughly parallel fashion, E_{PAH} varying from 5 to 20 per cent normal and oxygen consumption from 20 to 65 per cent normal.

Post mortem examination. The kidneys showed widespread tubular degeneration and necrosis with scattered, minute deposits of calcium. The proximal convoluted tubules were most involved. In many areas only tubular outlines remained recognizable and the tubular lumen was obliterated. The severity of the morphological changes was well-correlated with the degree of reduction in PAH extraction and oxygen consumption, but not with the urine volume.

DISCUSSION

These studies show that mercury bichloride in the dosage employed here will consistently produce severe renal tubular damage in the dog, and that initial transfusions of human blood will occasionally do so. They also suggest that appropriately timed second transfusions are much more likely to produce renal damage than the first, a view supported by other data (17, 18). Although the microscopic morphology indicated a difference in location and intensity of the damage caused by blood as opposed to that caused by mercury (in agreement with Oliver's hypothesis [1]), all affected animals from both groups had developed a similar functional impairment in regard to PAH extraction and inability either to make urine or to concentrate it. The very low renal oxygen consumption found after mercury poisoning can be attributed to the widespread tubular necrosis and marked degeneration of the non-necrotic epithelium, while the nearly normal values found following the human blood infusions can be attributed to a less massive necrobiosis and to a prominent increase in the number of leukocytes in the interstitial tissues.

These studies also show that at a time two or three days after the initial injury, marked renal circulatory derangements are not necessarily a concomitant of the tubular damage produced either by mercury or by human blood. In fact, a marked circulatory derangement was found only in two out of four animals given mercury and in only one out of three developing tubular impairment from human blood. When present, the circulatory abnormality was a reduction of renal blood flow to approximately one-half normal or less without any suggestion of alteration in the corticomedullary flow pattern. Indeed, no evidence of functioning intrarenal shunts was found in any of the animals.

The reductions in renal blood flow were not nearly so great as those reported by Bull, Joekes, and Lowe (6) in human anurics just before or after the anuric period. Their data, obtained by using the Fick method, indicated renal blood flows of from 0 to 20 per cent of normal.

The immediate factors responsible for the occurrence of renal ischemia in the two animals developing it after bichloride of mercury injections appeared to be dehydration and "shock level" blood pressure in dog IIC and severe dehydration with extreme hemoconcentration in dog IIB. Renal edema, which has been implicated as a cause of renal ischemia and anuria (19–21), was minimal in the dogs given mercury, but its presence was more obvious in the dogs given blood, particularly in dog IC. In this dog, severe renal edema was noted and was the only obvious factor on which blame could be laid for the presence of renal ischemia.

Finally, the studies show that in regard to the two experimental abnormalities produced herein, the urinary finding of extreme oliguria or actual anuria can be much better correlated with the

presence of renal circulatory impairment than with the presence or extent of tubular damage. The three dogs (IC, IIB, IIC) making little or no urine, were the same ones found to have moderately severe renal ischemia, while all the remaining dogs had a fair to good urinary minute volume and an essentially normal renal circulation. On the other hand, the demonstrated abnormalities in tubular function and morphology were almost identical in dogs IB and IC given human blood and also, in dogs IIB and IIC given mercury; yet the urinary minute volume in the former of each group was only moderately decreased, whereas it was *nil* in the latter of each group. Some years ago, it was stated (*22*) that the development of anuria following mercury bichloride poisoning was the result of complete loss of tubular impermeability with resultant glomerular filtrate-blood iso-osmotic equilibration. The presence of an essentially normal urinary volume (and renal circulation) in dog IID, having histological evidence of tubular necrosis quite as marked (see Figure 3) as that found in anuric dog IIC, suggests that filtrate-blood iso-osmotic equilibration is not the most important factor involved. Tubular luminal obstruction by casts and detritus has also been implicated as a cause of anuria by many authors including Maluf (*23*), Oliver (*24*), and Harrison, Bunting, Ordway, and Albrink (*25*). As judged from our sections prepared by routine histological technique, admittedly inferior to individual nephron dissection, there was moderate luminal obstruction in the kidneys subjected to mercuric chloride and minimal obstruction in the kidneys subjected to human blood. Of perhaps more significance was the finding of little or no recognizable difference in this respect between the kidneys of the urinating and those of the anuric dogs.

This combination of findings leads us to propose that anuria, when present "subacutely" in the dog following mercuric chloride injections or human blood transfusion, is not simply a direct result of severe tubular damage; but rather complete urinary suppression, as opposed to moderate oliguria, is dependent mainly on a superimposition of renal ischemia upon the existing nephron damage. The

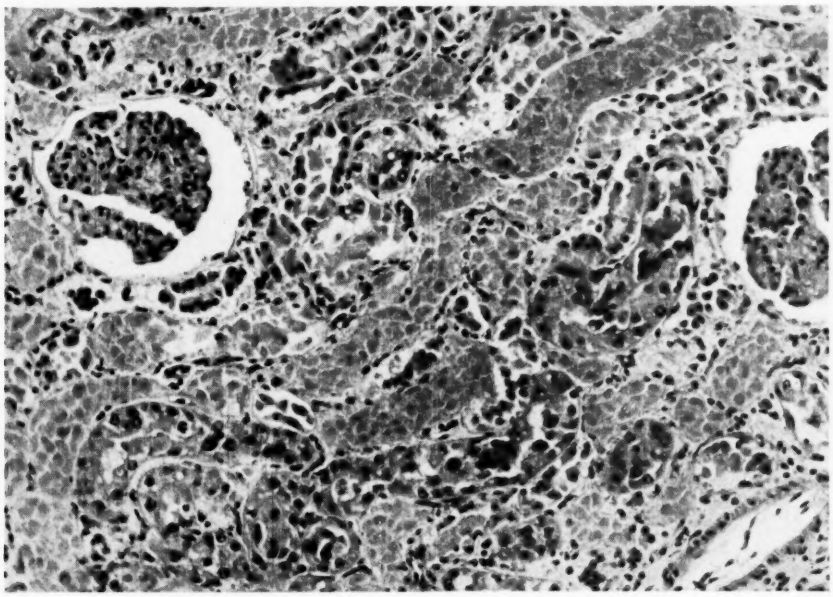

FIG. 3. PHOTOMICROGRAPH OF KIDNEY OF DOG IID GIVEN MERCURY BICHLORIDE FORTY-EIGHT HOURS PREVIOUSLY (H & E STAIN)

There is widespread tubular necrosis involving proximal tubules most severely. Note complete loss of structure with obliteration of lumina in some areas. Glomerular changes appear to be negligible.

renal ischemia may in turn be due, not directly to tubular damage, but to such sequelae thereof as renal edema or cast obstruction or it may result from such systemic derangements as shock or dehydration.

SUMMARY

1. The effects of intramuscular mercuric chloride injection and transfusions of human blood on renal tubular function, renal circulation, urinary volume and composition, and renal morphology in the dog were studied two to four days after the initial stimulus.

2. Mercuric bichloride consistently produced renal tubular damage, while human blood produced renal tubular damage occasionally after a first transfusion and in the only dog surviving a repeated transfusion.

3. Anuria was present in two out of four surviving animals having renal damage due to mercury poisoning, and one out of three having damage following human blood transfusion.

4. The renal circulation was essentially normal in all animals, save the anuric ones in which renal blood flow was reduced to 50 per cent of normal or less. As judged by renal arteriography, no functioning intrarenal shunts were present in any of the animals.

5. It is proposed that in the present experiments, the occurrence in three animals of anuria rather than moderate oliguria was mainly due to the superimposition of renal ischemia upon existing tubular damage.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Carl F. Schmidt for extending to them the use of the facilities of the Department of Pharmacology during the course of these studies and to Drs. C. F. Schmidt, J. K. Clark, and F. C. Wood for constructive criticisms of the manuscript.

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STUDIES OF MICROBIAL POPULATIONS ARTIFICIALLY LOCALIZED *IN VIVO*. I. MULTIPLICATION OF BACTERIA AND DISTRIBUTION OF DRUGS IN AGAR LOCI[1]

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(Submitted for publication August 5, 1953; accepted January 13, 1954)

Knowledge concerning the action of antimicrobial drugs on susceptible parasites has been gathered almost exclusively from the study of *in vitro* systems and from animal protection tests. *In vitro* systems represent a decaying environment which can only approximate for short periods of time and within narrow limits the complex biochemical and physiologic conditions obtaining *in vivo*. In contrast, within the animal host a continuous supply of nutriments is available to support microbial multiplication for prolonged periods of time, and may well permit greater physiologic heterogeneity among the parasites. *In vitro* systems do not generally include certain properties of the natural lesion such as necrosis, fibrosis, and parasites located within the cells. Likewise excluded from *in vitro* systems are certain important drug-host factors which may have a profound effect upon chemotherapeutic action. These include elimination or destruction of drug, binding by plasma proteins, and anatomicophysiologic barriers which may impede passage of drug from one body compartment to another (1).

Studies of the direct action of antimicrobial compounds on bacteria *in vivo* have been limited by the lack of suitable technics. Animal protection tests have not allowed precise control over the bacterial populations under study. An ideal technic would be one in which the microorganisms could be localized in an animal host in a manner in which the parasites would be subsisting directly upon nutriments supplied by the host but in which they would be available for removal for quantitative studies at desired intervals. Only a few attempts have been made to develop such technics. Eagle, Fleischman, and Musselman (2) have injected pneumococci and streptococci directly into the thigh muscle of the mouse and the testicle of the rabbit. With this method no attempt is made to ensure localization of the bacteria. Lurie inoculated phagocyted tubercle bacilli into the anterior chamber of the eye of the rabbit in order to study the responses of immune and non-immune animals (3). The same investigator has also injected animals subcutaneously with suspensions of virulent tubercle bacilli in melted agar and has implanted small collodion bags containing tubercle bacilli suspended in agar in the peritoneal cavities of rabbits (4). These agar inocula were well penetrated by the tissue fluids and supported growth of the tubercle bacilli *in vivo*.

In the present study the suitability of unenriched agar as a medium for isolating microorganisms growing *in vivo* was investigated further. A technic was devised whereby pathogenic bacteria were incorporated into the central portion of triple layered agar discs which were inserted into the peritoneal cavities of experimental animals for varying periods of time. The discs containing bacteria could be conveniently removed for quantitative study at appropriate intervals. In addition, the transfer of penicillin, streptomycin, chlortetracycline, and chloramphenicol across the agar to the parasites has been determined. A description of this technic which was originally suggested by the work of Frost (5), and the results obtained, constitute the basis of the present report.

MATERIALS AND METHODS

Preparation of agar. A 3 per cent suspension of fine granular agar (Difco Bactoagar) in distilled water, pH 6.0, in Erlenmeyer flasks was autoclaved at 250° F. for 30

[1] This study was aided in part by grants from Chas. Pfizer & Co., Brooklyn, N. Y.; the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.; and the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

[2] Postdoctorate Research Fellow, National Institutes of Health, U. S. Public Health Service during the period in which this study was conducted.

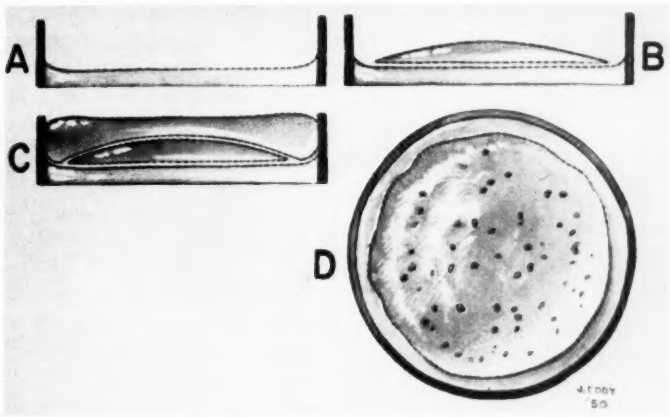

FIG. 1. STEPS IN THE CONSTRUCTION OF A TRIPLE LAYERED AGAR DISC

A, B, C. Cross sections of the disc showing the relationship of the three layers in the aluminum cylinder. D. Top view of a completed disc containing bacterial colonies.

minutes. The flasks were stored at 4° C. after the agar had solidified. At the time of an experiment, a flask of agar was placed in boiling water, and the melted agar was then maintained at the desired temperature in a water bath. In addition to the unenriched agar in distilled water, a small quantity of 3 per cent agar was prepared in buffered phosphate solution, pH 7.6. Ordinary 1.5 per cent beef heart infusion agar with or without added human serum or rabbit's blood was also used in certain experiments.

Preparation of agar discs. Agar was poured under sterile precautions into clean aluminum cylinders 7 mm. high and one mm. thick with an inside diameter of 22 mm. which were set in petri dishes resting on a cold surface. Triple layered agar discs containing inocula of bacteria were prepared as shown in Figures 1 and 2. The bottom layer consisted of 2 to 2.5 ml. of melted agar which filled the lower one-third to one-half of the cylinder and sealed the disc at its perimeter. Preliminary dilutions of culture were carried out in 0.85 per cent sterile saline, and a final dilution of 10^6 or 10^7 was made in agar at 45 to 50° C. in a water bath so that essentially no nutritive material was transferred. The inoculated agar was thoroughly mixed, and approximately 0.3 ml. was deposited from the tip of a warmed 2 ml. pipet upon the center of the hardened bottom layer. The agar of this central layer was led to within 2 to 2.5 mm. of the inner wall of the cylinder and did not come in contact with the cylinder. A final layer of 2 to 2.5 ml. of sterile agar was poured over the previous layers to fill the remaining volume of the cylinder.

In the completed disc, therefore, the central inoculated layer was completely sealed by sterile agar approximately 2 to 3 mm. thick. The whole procedure was accomplished as quickly as possible in order to minimize exposure of the microorganisms to the heat. The triple layered agar discs were used to study the growth of pathogenic bacteria in various animal hosts and *in vitro* in various media.

Single layered agar discs were prepared by delivering 4 or 5 ml. of agar into an aluminum cylinder and allowing the agar to solidify. These discs were used in studying the passage of drugs into agar *in vitro* and *in vivo*.

OBSERVATIONS

Bacterial growth in agar discs

The ability of unenriched 3 per cent agar to support bacterial growth was tested under a variety of conditions.

The following strains of bacteria were employed: *Diplococcus pneumoniae*, Type I; *Streptococcus pyogenes* (C203); *Streptococcus zymogenes; Staphylococcus au-*

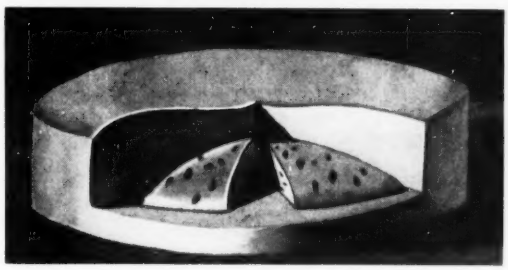

FIG. 2. DIAGRAMMATIC REPRESENTATION OF A COMPLETED DISC CONTAINING BACTERIAL COLONIES

The metal ring has been removed and the disc has been cut open to show the internal construction.

reus; Klebsiella pneumoniae, Type A-D; *Salmonella typhosa; Brucella melitensis; Bacillus anthracis;* and *Mycobacterium tuberculosis* (H37Rv). Cultures were incubated 18 or 24 hours at 37° C. in infusion broth or 2 per cent blood broth prior to use, except that *Br. melitensis* culture was a 96-hour growth in trypticase soy broth and *M. tuberculosis* culture was a 96-hour growth in liquid oleic acid-albumin medium.

Bacterial density and homogeneity of the cultures were determined by conventional methods at the start of each experiment, and bacterial growth was studied as follows:

(a) Cultures streaked on the surface of buffered and unbuffered agar, and pour plates of 10^6 dilutions of culture in sterile saline were sealed against drying and were incubated at 37° C. for 48 hours to 5 days.

(b) Triple layered agar discs in which the central layer consisted of approximately 0.3 ml. of agar comprising a 10^6 or 10^7 dilution of culture were incubated at 37° C. for 24 to 48 hours in deep petri dishes flooded with 60 to 100 ml. sterile saline and with infusion broth or 5 per cent serum broth, respectively. Discs inoculated with *Br. melitensis* were incubated in trypticase soy broth for periods up to five days.

(c) Triple layered agar discs similarly prepared were inserted into the peritoneal cavities of rabbits, guinea pigs and cats. The animals were anesthetized with ether, chloroform, or intravenous pentobarbital, and the peritoneal cavity was opened through an abdominal incision using aseptic surgical technic. The discs with their surrounding aluminum rings were directed into various parts of the peritoneal cavity in a manner to ensure maximum contact with peritoneal surfaces. The wounds were closed, and the animals were permitted to recover consciousness. The discs were removed through the same or a separate incision 24 hours later for examination. If no bacterial colonies were observed the discs were reinserted for an additional 24-hour period or longer. Discs which were found to contain colonies were transferred to sterile petri dishes and were opened with a sterile spatula. The organisms within the discs were subcultured on appropriate media, and their identity was confirmed by study of morphology, gram stain, and agglutination in appropriate antiserum. Peritoneal cultures were made at the times of insertion and removal of the agar discs in order to determine whether contamination of the peritoneal cavity had occurred during the procedure.

Unenriched agar. Agar without added nutrient permitted only minimal growth of a few strains of bacteria. When cultures were streaked on the surface of 3 per cent agar plates only a few, very tiny and irregular colonies barely visible to the unaided eye were apparent after 24 hours of incubation for staphylococci, enterococci, and *S. typhosa.* Somewhat larger colonies of *Kl. pneumoniae* and *B. anthracis* were noted. No discernible colonies of C203 streptococci, type I pneumococci, or *Br. melitensis* were detected on microscopic examination of the plates even after five days of incubation. No difference was noted with agar which had been buffered to pH 7.6. In the pour plates and in the triple layered agar discs which were incubated in saline, *Kl. pneumoniae* was the only microorganism which regularly formed colonies. At 24 hours these colonies were 100 to 150 microns in size and could be detected only with the microscope, while at 48 hours minute colonies could be seen in the depths of the agar with the unaided eye. *M. tuberculosis* was not tested under these conditions.

Enriched agar. In contrast to the meager growth in the unenriched agar, colony development was luxuriant and regular in triple layered agar discs incubated in broth or 5 per cent serum. Numerous large colonies were noted for all species and were comparable to colonies appearing in conventional pour plates of highly nutritive agar. Colonies of *Br. melitensis* were first visible at 72 hours while those of the other species were apparent at 12 to 24 hours. The tubercle bacillus was not tested.

Agar discs in vivo. Three to five triple layered agar discs inoculated with bacteria were implanted intraperitoneally in each of two rabbits, guinea pigs, and cats, respectively, for each of the aforementioned organisms except that the tubercle bacillus was studied only in rabbits. *Kl. pneumoniae, S. typhosa,* and *B. anthracis* consistently grew numerous large colonies in the depths of the discs within 24 hours in all animals (Figure 3). Growth of the gram positive cocci and *Br. melitensis* was less regular. The staphylococcus formed numerous visible but very small colonies (Figure 4). The C203 streptococci, type I pneumococcus, and enterococcus regularly formed microscopic colonies 50 to 100 microns in size within 24 to 48 hours in all discs and a small to moderate number of larger colonies visible to the unaided eye in the majority of discs. *Br. melitensis* formed many microscopic colonies, 30 to 50 microns, in the depths of the discs in 72 hours, but the colonies did not attain visible size even after five days of incubation. No colonies of *M. tuberculosis* were noted in discs which had remained in rabbits for as long as 20 days. In retrospect, however, the

conditions of the experiment were of a sort known to be unfavorable for the growth of human tubercle bacilli.

Contamination of the peritoneal cavity by the organism which had been incorporated in the agar occurred in about 25 per cent of trials. This was most frequently encountered with *Kl. pneumoniae* and *S. typhosa* and occurred more often in guinea pigs than in rabbits or cats. In addition, contamination of the peritoneal cavity with *B. proteus* sometimes occurred. Occasionally peritoneal cultures were found to be positive for the organism under study immediately after a number of discs had been inserted and before bacterial multiplication had begun.

Peritoneal reaction to implanted discs

The tissue reaction to the agar discs was carefully noted at the time of removal from the animals. In addition, discs which had been inoculated with *M. tuberculosis* and which had been implanted in rabbits for periods of one to 20 days were fixed in Bouin's solution immediately on removal. These were sectioned perpendicularly to the flat surface and were stained with hematoxylin and eosin, Giemsa's stain, and carbol fuchsin stain for microscopic examination.

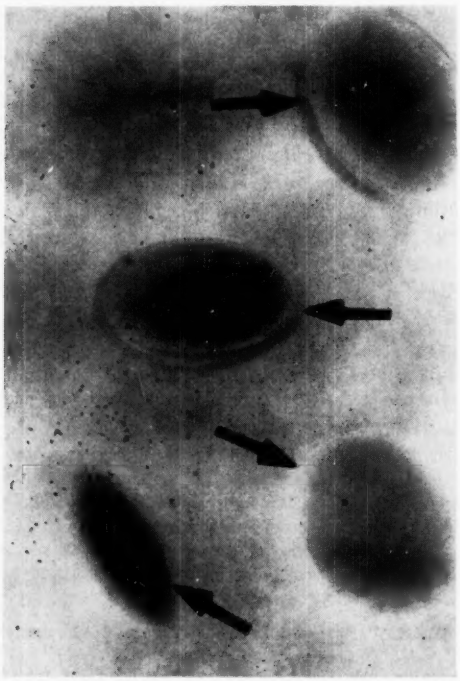

FIG. 3. FOUR COLONIES OF SALMONELLA TYPHOSA (INDICATED BY ARROWS) IN THE DEPTHS OF AN AGAR DISC REMOVED FROM THE PERITONEAL CAVITY OF A GUINEA PIG AFTER 24 HOURS OF INCUBATION (110×)

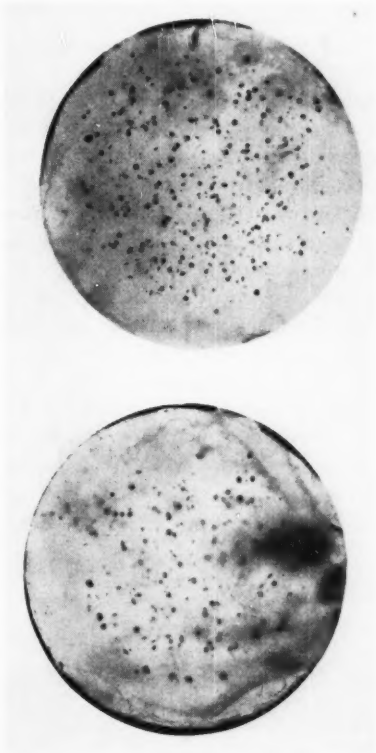

FIG. 4. TRIPLE LAYERED AGAR DISCS INOCULATED WITH STAPHYLOCOCCUS AUREUS AND INCUBATED 24 HOURS IN THE PERITONEAL CAVITY OF A GUINEA PIG, SHOWING COLONY FORMATION IN THE DEPTHS OF THE DISC

A conspicuous tissue reaction to the agar discs implanted in rabbits occurred not only with discs inoculated with bacteria but also with plain agar discs. Within 24 hours the discs were completely enveloped in a thin fibrinous film which increased in thickness to a millimeter or more by 48 to 72 hours. The film of exudate could be easily stripped away from the agar, revealing the smooth surface of the disc.

Microscopic examination of sections of agar discs inoculated with tubercle bacilli and removed from rabbits revealed the exudate to consist pre-

dominantly of fibrin and polymorphonuclear leukocytes with a smaller proportion of round cells at 24 hours. After 48 hours increasing numbers of mononuclear cells and fibroblasts were noted. Although inflammatory cells were present in large numbers in the exudate on the surface of the agar and had infiltrated the agar along small cracks in the surface or along the interfaces between the different layers, the cells were not able to penetrate the substance of the agar (Figure 5). After seven to ten days small bits of agar had become broken off the surface of the discs and were completely surrounded by organizing exudate.

The tissue reaction in guinea pigs was of the same type as that of the rabbits, but it was less intense and the exudate was not so thick. In cats, on the other hand, little or no exudate was found surrounding the discs, but the discs were often wrapped in hyperemic omentum. In rabbits and guinea pigs the omentum less frequently enveloped the discs in the peritoneal cavity.

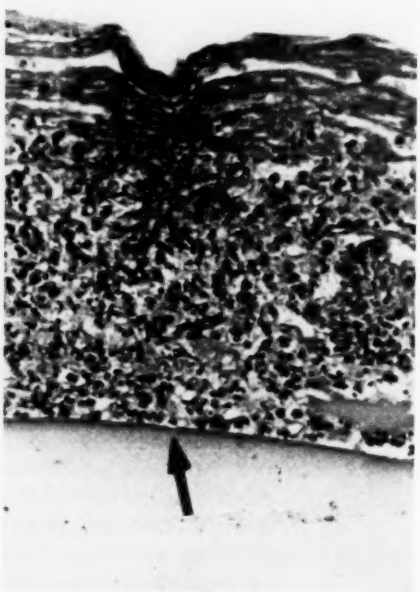

FIG. 5. SECTION THROUGH AN AGAR DISC REMOVED FROM THE PERITONEAL CAVITY OF A RABBIT AFTER 48 HOURS OF INCUBATION SHOWING THE NATURE OF THE EXUDATE UPON THE SURFACE OF THE DISC

The cells have not penetrated the surface of the agar (indicated by arrow). Hematoxylin and eosin stain (430 ×)

Multiplication of S. typhosa in agar discs in the cat

Triple layered discs were prepared which contained exactly 0.3 ml. of agar consisting of a 10^7 dilution of 18-hour culture of *S. typhosa* of known bacterial density. Seven discs were introduced into the peritoneal cavity of a cat anesthetized with intravenous sodium pentobarbital and the edges of the laparotomy wound were drawn together with temporary sutures which could be opened and closed at will. One disc was removed from the animal at 0, ½, 1, 2, 3, and 4 hours, respectively, while the seventh disc remained inside for 24 hours. On removal each disc was transferred to a sterile mortar containing a small amount of sterile sand in 6 ml. of sterile saline and was ground under sterile precautions for 10 minutes. An aliquot was then removed and appropriate dilutions were made in saline. Infusion agar pour plates of these dilutions were incubated for 24 hours and the total numbers of bacilli present in the discs were computed.

Three discs similarly prepared were incubated *in vitro* in infusion broth for 24 hours. The number of colonies present in these three discs and in the disc which had remained in the peritoneal cavity for 24 hours yielded the average number of bacilli per disc at the start of the experiment. These discs further served as controls on the possible destructive effect on the bacteria of the grinding with sand. Control discs were similarly ground and plated after four hours of incubation *in vitro* in the dry state and in petri dishes flooded with saline, respectively. These discs served to control possible growth of typhoid bacilli in unenriched agar.

Growth curves of *S. typhosa* in cats have been shown in Figure 6. The microorganisms promptly entered the logarithmic growth phase within four hours in the intraperitoneal discs while no significant multiplication occurred in the control discs.

Penetration of drugs in 3 per cent agar

Penetration in vitro. The rate of diffusion of penicillin,[3] streptomycin,[4] chlortetracycline [4] and chloramphenicol [5] between 3 per cent agar and broth was studied under two sets of circumstances:

(a) Five ml. quantities of broth containing a known concentration of drug were exposed to single layered agar discs of equal volume which had been finely diced with a sterile spatula;
(b) Single layered 5 ml. agar discs containing a known concentration of drug were finely diced and were exposed to equal volumes of plain broth.

[3] Supplied as crystalline penicillin G and as streptomycin sulfate by Chas. Pfizer & Co., Brooklyn, N. Y.

[4] Supplied as Aureomycin Hydrochloride by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

[5] Supplied as Chloromycetin® by Parke, Davis and Co., Detroit, Mich.

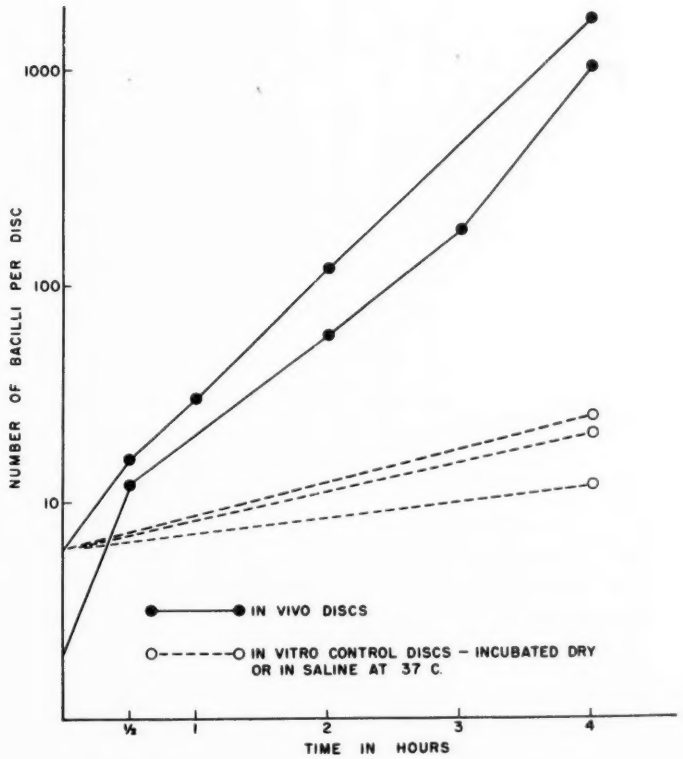

FIG. 6. RATE OF GROWTH OF S. TYPHOSA IN AGAR DISC IN PERITONEAL CAVITY OF A CAT

Two series of five large test tubes containing these respective combinations of broth, agar, and drug were incubated at 37° C. after vigorous shaking. The broth was removed by pipet as completely as possible from one tube in each series at ½, 1, 2, and 3 hours, respectively, for assay of drug concentration. The initial concentration of drug in broth and in agar at the start of each experiment was 20 micrograms per ml. for penicillin, 50 micrograms per ml. for streptomycin, and 200 micrograms per ml. for chlortetracycline and chloramphenicol.

To control the effect which heat may have in reducing the antibacterial potency of these compounds, broth which contained drug was exposed for comparable periods of time in a water bath to temperatures equivalent to those to which the drug was exposed in the melted agar. In addition a standard solution of drug in broth was incubated concurrently with the two series of tubes for each compound. Aliquots of the standard were removed at the same intervals. All specimens of broth were immediately frozen and were stored at − 18° C. Microbiologic assay was carried out by means of a fractional dilution technic capable of detecting smaller changes in drug concentrations than the conventional twofold serial dilution method (6).

Three to six determinations were made for each drug in order to control such variables as the degree of dicing of the agar and agitation of the tubes. The results have been recorded in Table I and in Figure 7. When diced 3 per cent agar discs were added to an equal volume of broth containing penicillin, streptomycin, chlortetracycline, or chloramphenicol, the concentration of drug in the broth fell progressively, indicating the transfer of these drugs into the agar. Allowing for deterioration of drug due to heat as indicated by loss of potency of the standard, the concentration of drug in the broth fell within 30 to 60 minutes to a value approximately one-half the initial concentration or one-half the concentration of the standard after a comparable period of incubation. Conversely when diced agar discs containing known concentra-

TABLE I

Rate of diffusion of penicillin, streptomycin, chlortetracycline and chloramphenicol between 3 per cent agar and broth at 37° C.

	Broth concentrations in micrograms/ml.														
	Standard					Agar + Drug exposed to broth					Broth + Drug exposed to agar				
Trial	0	½	1	2	3	0	½	1	2	3	0	½	1	2	3 H.
Penicillin															
1	20		13	10	10	0	5	5	7	5	20	12	6	5	7
2	20	20	20	20	13	0	7	7	10	10	20	13	13	13	13
3	20	13	13	13	13	0	9	9	9	9	20	9	9	9	9
Av.	20	16	15	14	12	0	7	7	9	8	20	11	9	9	9
Streptomycin															
1	50	40	40	40	50	0	7	15	10	10	50	30	25	20	20
2	50	50	50	50	38	0	19	12	12	19	50	30	30	30	25
3	50		37	25	25	0		12	12	12	50		12	18	18
Av.	50	45	42	38	38	0	13	13	11	14	50	30	22	23	21
Chlortetracycline															
1	200		200	200	150	0	30	30	45	30	200	120	120	120	90
2	200	200	200	200	100	0	51	51	51	51	200	160	133	100	100
3	200	200	200	200	160	0	40	60	60	60	200	100	100	76	76
4	200	122	100	157	122	0	20	24	32	40	200	125	100	125	66
5	200	165	200	200	165	0	25	40	33	25	200	265	132	100	132
6	200	200	264	200	160	0	40	68	68	68					
Av.	200	177	194	193	143	0	34	46	48	46	200	154	117	104	93
Chloramphenicol															
1	200		200		200	0	100	100	100	100	200	132	132	132	100
2	200				200	0	100	100	100	100					
3	200	200	200	200	200	0	80	100	100	100	200	132	100	100	100
4	200				200	0	66	80	80	80	200	132	132	132	132
Av.	200	200	200	200	200	0	86	95	95	95	200	132	121	121	111

tions of these four drugs were added to equal volumes of plain broth, the concentration of drug in the broth rose progressively. Within a period of 30 to 60 minutes the drug in the broth approached a concentration approximately one-half the initial concentration of drug in agar or one-half the concentration of the standard after a comparable period of incubation.

In the case of penicillin and chloramphenicol, almost exactly equal distribution of drug between agar and broth was achieved in this *in vitro* system. For streptomycin and chlortetracycline, however, the concentrations in the broth which had been exposed to agar containing drug were only 50 per cent of those which would have obtained had equal distribution of these two drugs between agar and broth occurred. This yielded a factor of two for penicillin and chloramphenicol and a factor of four for streptomycin and chlortetracycline, respectively, in determining the original concentration of these drugs in the agar by this method.

Differences in the values in Table I for successive determinations generally corresponded to a difference of only one or two dilutions in the fractional dilution assay which is within the limits of error of this test. These results validated the equilibration method of estimating the drug content of the agar discs.

Penetration in vivo. Rabbits or cats were weighed and were anesthetized with intravenous sodium pentobarbital. Six to eight 5 ml. agar discs were introduced into the peritoneal cavity with care to ensure contact of the flat surfaces of the agar discs with the peritoneum and to prevent apposition of discs with each other. After all discs had been inserted the animals received a large intravenous dose of penicillin, streptomycin, or chlortetracycline or a large oral dose of chloramphenicol. Specimens of blood were obtained from the exposed femoral artery or by intracardiac puncture and agar discs were removed 1, 2, 4, 6, 8, 24, and 48 hours after drug administration. In animals receiving drug by the intravenous route, additional specimens were collected at 5, 15, and 30 minutes. The blood was allowed to clot for one hour at 4° C. and the serum was removed after centrifugation.

The discs were diced with a sterile spatula and were transferred to large test tubes containing an equal volume of broth. After vigorous shaking the tubes were incubated at 37° C. for one hour to permit equilibration of drug. All specimens of serum and broth were frozen and were stored at −18° C. until time of assay. Microbiologic assay was carried out by means of the conventional two-fold serial dilution technic, and the concentration of drug in the agar discs was taken to be twice that obtained in the broth for penicillin and chloramphenicol and four times for streptomycin and chlortetracycline. Standard solutions of drug in broth were assayed concurrently with the unknown specimens.

After the six or eight-hour specimens had been obtained the wounds were closed and the animals were allowed to regain consciousness. The animals were reanesthetized and new incisions were made at 24 and 48 hours. In order to prevent dehydration, the animals received by gavage 50 ml. of 10 per cent glucose in one per cent saline at 4 and 24 hours after the start of the experiment.

The rate of penetration of agar discs within the peritoneal cavity by penicillin, streptomycin, chlortetracycline, and chloramphenicol and the relation of the serum concentrations to the concentrations in agar have been shown in Figure 8. High concentrations were readily attained in the agar discs. After intravenous administration to rabbits, penicillin and streptomycin rapidly accumulated in the agar discs, and concentrations equal to or exceeding the corresponding serum concentrations were achieved between one and two hours for penicillin and between one and four hours for streptomycin, respectively. In cats given chlortetracycline intravenously, measurable amounts of drug were present in agar discs removed from the peritoneal cavity 30 minutes after injection. The concentrations rose more slowly than those of penicillin and streptomycin, however, and did not attain the same magnitude as the serum concentrations. Measurable concentrations of chloramphenicol were present in discs removed from cats one or two hours after oral administration of this drug but remained below those in the serum. Concentrations of drug in the agar discs fell slowly, and measurable concentrations of all drugs except penicillin were still present 24 hours after administration of the single doses.

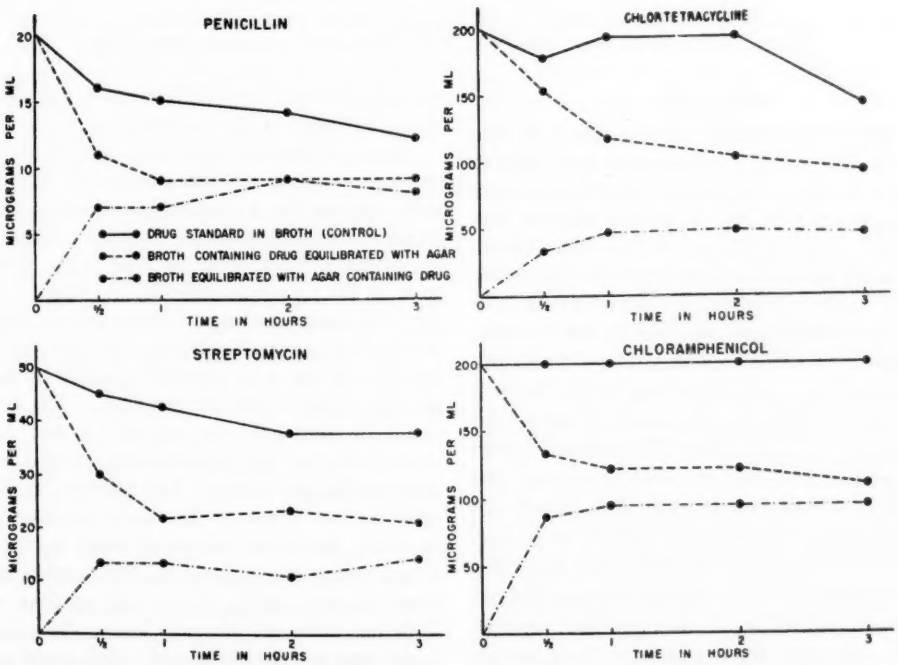

FIG. 7. RATE OF DIFFUSION OF PENICILLIN, STREPTOMYCIN, CHLORTETRACYCLINE AND CHLORAMPHENICOL BETWEEN BROTH AND 3 PER CENT AGAR IN VITRO AT 37° C.

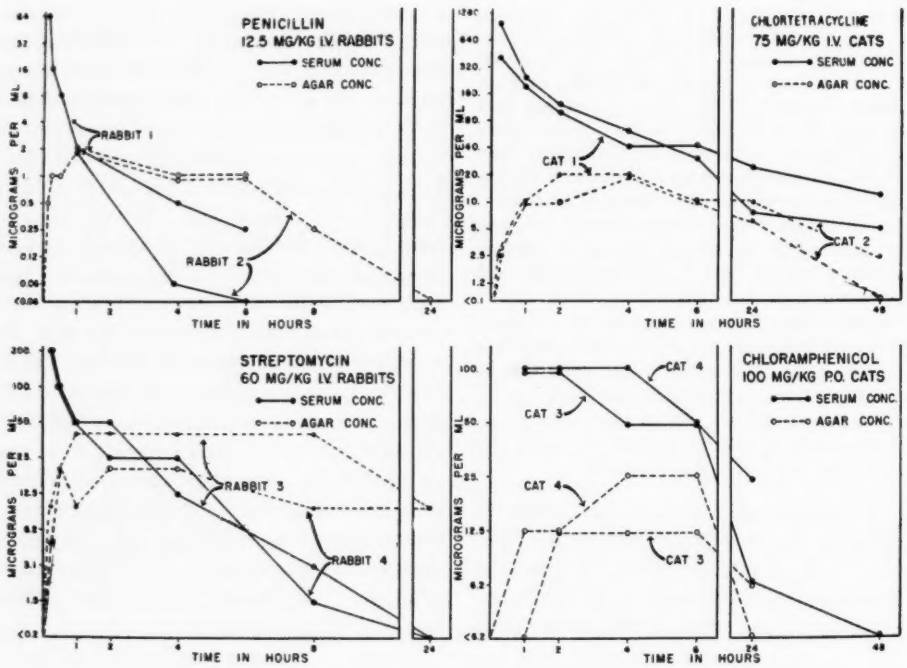

FIG. 8. RELATION OF CONCENTRATIONS OF PENICILLIN, STREPTOMYCIN, CHLORTETRACYCLINE AND CHLORAMPHENICOL IN INTRAPERITONEAL AGAR DISCS TO SERUM CONCENTRATIONS

DISCUSSION

The above observations indicate that it is possible by means of the triple layered agar discs to localize a microbial population effectively within the experimental animal in such a manner that quantitative determinations of bacterial growth can be made at regular intervals. In this situation the microorganisms were found to be subsisting upon nutriments supplied by the extracellular fluid of the host in a state more closely approximating true parasitism than would be possible *in vitro*. While it is true that certain species of pathogenic bacteria such as *Kl. pneumoniae* were able to grow to a limited degree on 3 per cent agar to which no nutriment was added, this growth was not comparable to the luxuriant growth of the bacteria which occurred in the agar discs implanted in the peritoneal cavity of the experimental animal.

The bacteria growing in the agar discs were effectively protected from attack by host macrophages and yet were exposed to the action of chemotherapeutic drugs which quickly passed through the host body fluids to penetrate the agar discs implanted in the peritoneal cavity.

The triple layered agar discs used in these experiments were easy to construct in the laboratory, and with practice a considerable number could be prepared in a short time. Thermal killing of the organisms did not occur as long as the temperature of the agar was carefully regulated. Three per cent unenriched agar constituted an adequate matrix for bacterial multiplication, and the transparency of the agar enabled colonies to be seen directly in the depths of the discs. The bacteria could be recovered for subculture and further study such as drug-susceptibility determinations after incubation *in vivo*. The rubbery consistency and the flat shape of the discs permitted easy handling, and discs remained intact in the peritoneal cavity for as long as three weeks and in broth in petri dishes for as long as four weeks. Only an occasional disc was lost in the peritoneal cavity due to crumbling, and a disc could usually be recovered whole even when it had slipped out of the encircling aluminum cylinder.

One deficiency of the agar disc technic was that the enclosed organisms were not always completely confined and peritoneal contamination sometimes resulted. The incidence of contamination varied with: the care with which the discs were prepared and introduced into the animals; the number of discs inserted and the size of the bacterial inoculum; the time of incubation within the animals; and the organism which was selected. As proficiency in technic was acquired the frequency of peritoneal contamination diminished. In the few instances in which the peritoneal culture was found to be positive immediately after the discs containing bacteria had been inserted, it is not unlikely that contamination of the exterior of the discs may have occurred from aerosol created during the pouring of the middle layer. Nevertheless, the efficacy of agar in providing an effective medium for localizing large numbers of bacteria was demonstrated by the fact that discs containing 100 to 150 colonies of staphylococci or typhoid bacilli were removed from animals after 24 hours of incubation and the peritoneal cultures of these animals were sterile. Moreover, discs containing many colonies of bacteria remained in petri dishes flooded with broth for as long as 48 hours without contamination of the surrounding liquid medium.

The chief shortcoming of the agar discs in studying drug-host-parasite relationships in experimental animals is that they are an artificial and unphysiologic means of endeavoring to reproduce the conditions present during infection. The end-result produced by a drug on a microbial population *in vivo* involves the interaction of a number of factors which have to do with both drug-host and drug-parasite relationships (1). Nevertheless the present technic makes it possible to separate drug-bacteria reactions within a host from the host's own cellular reactions and may be helpful in defining the respective roles of cellular defense and drug effect in the study of a particular infection.

It is also believed that the foregoing experimental method will have a degree of usefulness in the study of other biologic phenomena pertaining to chemotherapy and the behavior of bacterial populations in animal hosts. By mixing bacteria in the middle layer of the disc with purulent exudate or caseum, for example, it may be possible to determine the degree to which these products of natural infection compromise drug action. The formation of a thick, fibrinocellular film about the agar discs in rabbits affords additional means of investigating the ability of antimicrobial drugs to penetrate such membranes. In the present study it is significant that penicillin given in large doses to rabbits more readily penetrated the exudate encasing agar discs in the peritoneal cavity than solid fibrin clots implanted subcutaneously as reported elsewhere (7).

Finally, the use of this technic promises to serve as a critical test of the ability of new chemotherapeutic substances to pass the several anatomico-physiologic barriers which exist in the host between the site of administration of a drug and its locus of antimicrobial action in the extracellular fluid. In this way the use of the agar discs may help to explain certain discrepancies of drug action *in vivo* and *in vitro*. Such an investigation into the differences in antityphoidal action of chlortetracycline and chloramphenicol is the subject of a separate report (8).

SUMMARY

A method has been outlined whereby a bacterial population has been successfully localized in the peritoneal cavity of an animal host for varying periods of time. The bacteria were localized in the central portion of triple layered 3 per cent agar discs and were completely isolated by surrounding transparent agar. As no nutriment was added to the agar, the bacteria were dependent for growth on nutritive substances from the surrounding tissue fluids and were able to exist in a state more closely approximating true parasitism than is possible *in vitro*. The tissue reaction to the agar discs in the peritoneal cavity resembled a foreign body reaction. Exudate containing inflammatory cells lined the surface of the discs, but the cells were not able to penetrate the substance of the agar.

Pathogenic bacteria were tested for their ability to grow in several animal species in this situation. Staphylococci, typhoid bacilli, Friedlander's bacilli, and anthrax bacilli grew luxuriantly in agar discs in rabbits, guinea pigs, and cats, although growth had been negligible in unenriched agar *in vitro*. Streptococci, enterococci, pneumococci, and brucella also grew well in the agar discs *in vivo*, although the colonies sometimes attained only micro-

scopic size. Contamination of the peritoneal cavity occurred in less than 25 per cent of trials.

The agar discs were well penetrated *in vitro* and *in vivo* by penicillin, streptomycin, chlortetracycline, and chloramphenicol. It is expected that this method will be useful in assessing the direct effect of antimicrobial compounds in the extracellular fluids of animal hosts and in studying other biologic phenomena.

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STUDIES OF MICROBIAL POPULATIONS ARTIFICIALLY LOCALIZED *IN VIVO*. II. DIFFERENCE IN ANTITYPHOIDAL ACTIVITIES OF CHLORAMPHENICOL AND CHLORTETRACYCLINE [1]

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(Submitted for publication August 5, 1953; accepted January 13, 1954)

It has long been recognized that only a few compounds which exhibit antimicrobial activity *in vitro* are capable of manifesting comparable activity when introduced into mammalian hosts. The different pharmacologic and microbiologic factors which may be responsible for chemotherapeutic failure have been reviewed elsewhere (1, 2). In general, however, if a particular drug is capable of exerting powerful antimicrobial action *in vivo* against certain pathogens, that drug will be similarly effective against all pathogenic bacteria which are inhibited by low concentrations of the drug *in vitro*. Infections which have been notable exceptions to this generalization include typhoid fever, brucellosis, and tuberculosis. Although the causative agents have been inhibited *in vitro* by low concentrations of a number of drugs of proved value in other infections, only a few compounds have been significantly effective against these diseases in humans or experimental animals. Streptomycin, for example, has inhibited *Salmonella typhosa in vitro* in concentrations comparable on a weight basis to those of chloramphenicol, yet the former drug has been notably ineffective in the treatment of typhoid fever (3). Similar results have been noted for streptomycin in brucellosis (3) and for subtilin in experimental infections with *Mycobacterium tuberculosis* (4).

A more recent example of pronounced disparity of drug action *in vitro* and *in vivo* has been the virtual ineffectiveness of chlortetracycline in the treatment of typhoid fever in humans. Both chlortetracycline and chloramphenicol have been demonstrated to be highly active in relatively low concentration against a wide spectrum of pathogenic microorganisms including *S. typhosa* (5, 6), and both drugs have been found to be effective in the treatment of a large number of infections including intracellular infections other than typhoid, such as scrub and murine typhus and acute brucellosis (7–9). While chloramphenicol has been highly successful in the treatment of typhoid, chlortetracycline has been only negligibly effective in this disease (10).

Possible explanations for this phenomenon have been the following: (a) Conventional doses of chlortetracycline have failed to provide sufficient amounts of the drug or its active principle in the tissue fluids; (b) Chlortetracycline is rendered ineffective in the body because of the chemical or anatomic nature of the lesion produced by *S. typhosa;* and (c) Typhoid bacilli subsisting in the host are less susceptible to the action of chlortetracycline than typhoid bacilli growing *in vitro*.

The purpose of the present study was to investigate the suppressive effects of chlortetracycline and chloramphenicol on *S. typhosa* localized within experimental animals by means of small agar discs and to compare the results with the antityphoidal activities of the two compounds measured *in vitro*.

MATERIALS AND METHODS

Drug-susceptibility determinations in vitro. Two-fold serial dilutions in infusion broth and in 20 per cent serum broth, respectively, were made of standard solutions of chlortetracycline [3] and chloramphenicol [4] having an ini-

[1] This study was aided in part by grants from Chas. Pfizer & Co., Brooklyn, N. Y.; the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.; and the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

[2] Postdoctorate Research Fellow, National Institutes of Health, U. S. Public Health Service during the period in which this study was conducted.

[3] Supplied as Aureomycin Hydrochloride by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

[4] Supplied as Chloromycetin® by Parke, Davis and Co., Detroit, Mich.

tial concentration of 200 micrograms per ml. in distilled water. To the 0.5 ml. contents of each tube in the dilution series was added an equal volume of 10^6, 10^8, or 10^7 dilution of 18-hour culture of *S. typhosa* in infusion broth. The final concentrations of drug in each series of tubes ranged from 1:2 through 1:1024 of the original standard solution. Each series of tubes was incubated 24 hours at 37° C., and the end point was the concentration of drug in the first tube in which there was no visible bacterial growth. In order to determine whether the drugs had rendered the culture sterile, the contents of each tube showing no visible growth were subcultured in agar and broth, respectively. Bacterial homogeneity and density of the culture were determined by conventional methods in each experiment.

Drug-parasite reactions in vivo. Triple layer discs of 3 per cent unenriched agar were constructed under sterile conditions in which the central layer was completely encased by transparent agar and consisted of approximately 0.3 ml. of a 10^7 dilution of 18-hour culture of *S. typhosa*. Preliminary dilutions of culture were made in 0.85 per cent sterile saline, and the final dilution was made in agar at 45 to 50° C. in a water bath. Additional discs containing 4 or 5 ml. of sterile agar were used in assaying drug content of agar *in vivo*. This method has been described in detail (11).

Mongrel cats were weighed and were anesthetized with intravenous sodium pentobarbital. The abdomen was shaved and cleansed with tincture of iodine and alcohol, and a laparotomy incision was carried out using aseptic technic. Two freshly prepared discs containing typhoid bacilli and 6 or 7 discs of plain agar were inserted into the peritoneal cavity and were placed so that the discs were in close contact with the peritoneal surfaces but were not in apposition to each other. The wound edges were approximated with long silk sutures so that the incision could be reopened to remove the discs and closed again at intervals as desired.

After the discs had remained *in vivo* for three or four hours and bacterial multiplication within the agar had entered the logarithmic phase, the animals were given single doses of chlortetracycline either intravenously or orally, or single oral doses of chloramphenicol, respectively. Animals receiving drug orally had been fasted for 18 hours previously. Blood specimens were withdrawn under sterile precautions from an exposed femoral artery or by cardiac puncture and uninoculated discs were removed from the peritoneal cavity simultaneously at 1, 2, 4, 6, 24, and 48 hours after administration of drug. Additional specimens were taken 15 minutes after intravenous doses. Microbiologic assay of the blood serum and agar discs for drug content was carried out using a conventional two-fold serial dilution technic. Standard solutions of each drug were assayed concomitantly with the unknown specimens. The details and validity of this method and the penetrability of agar by the two drugs have been demonstrated in a previous study (11).

After the necessary specimens had been obtained at six hours, the wounds were sutured and the animals were allowed to regain consciousness. Fifty ml. of dextrose solution was given by stomach tube at this time to prevent dehydration. The cats were reanesthetized and new incisions were made to recover the agar discs at 24 and 48 hours. At these times the discs which had been inoculated with typhoid bacilli were also removed and were examined for colony formation in the depths of the agar. If no colonies were visible at 24 hours the discs were reinserted and were examined again at 48 hours. If no colonies were visible at 48 hours the discs were transferred to deep petri dishes containing infusion broth and were incubated *in vitro* 24 to 48 hours to determine whether sterilization had occurred.

Discs which were removed from the cats and found to contain visible colonies were opened with a sterile spatula and the organisms were subcultured on agar. Identity of the bacteria was confirmed by morphologic study, gram staining and agglutination in *S. typhosa* anti-serum. All animals were sacrificed after 48 hours. Other discs inoculated with typhoid bacilli were introduced into cats which did not receive antimicrobial drug and were incubated in infusion broth or sterile saline in large petri dishes to serve as controls of the ability of *S. typhosa* to grow in unenriched agar in animals and *in vitro*.

RESULTS

Drug-susceptibility of S. typhosa in vitro

Parallel determinations were made of the minimum inhibitory concentrations of chlortetracycline and chloramphenicol for different sized inocula of *S. typhosa* in infusion broth and in 20 per cent serum broth, respectively. The results have been shown in Table I. The minimum inhibitory concentrations of chlortetracycline ranged from 0.4 to 12.5 micrograms per ml. while those of chloramphenicol ranged from 0.4 to 6.2 micrograms per ml. These concentrations were proportional to the size of the inoculum, but there was seldom more than a two-fold increase in the quantity of drug required as the inoculum size increased ten-fold. The median minimal inhibitory concentrations of chlortetracycline at 24 hours were two to four times as high as those of chloramphenicol on a weight-volume basis. Inhibition of *S. typhosa* by both compounds was reversible and nonsterilizing as demonstrated by the fact that typhoid bacilli grew readily when subcultured from the tubes which showed no visible bacterial growth in the dilution series.

Drug-susceptibility of S. typhosa in vivo

The relationship between serum concentrations of drug and the suppressive effects on typhoid ba-

TABLE I

Parallel determinations of susceptibility of S. typhosa to chlortetracycline and chloramphenicol

DAY	NUMBER OF BACILLI PER ML 18 HOUR CULTURE X 10^8	MINIMAL INHIBITORY CONCENTRATION MICROGRAMS PER ML											
		CHLORTETRACYCLINE						CHLORAMPHENICOL					
		INFUSION BROTH			20% SERUM BROTH			INFUSION BROTH			20% SERUM BROTH		
		INOCULUM SIZE			INOCULUM SIZE			INOCULUM SIZE			INOCULUM SIZE		
		10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7
1	470	6.2	6.2	3.1				1.6	1.6	0.8			
2	810	12.5	6.2	6.2				1.6	1.6	1.6			
3	180	6.2	6.2	3.1	3.1	1.6	1.6	1.6	0.8	0.8	1.6	0.8	0.8
4	660	3.1	3.1	1.6	6.2	1.6	0.4	1.6	0.8	0.8	0.8	0.8	0.4
5	600	6.2	3.1	0.8	6.2	3.1	0.8	6.2	1.6	0.8	0.8	0.8	0.4
6	550	12.5	6.2	6.2	12.5	3.1	1.6	1.6	1.6	0.8	1.6	1.6	1.6
7	600	12.5	12.5	12.5				1.6	1.6	1.6			
8	630	3.1	1.6	1.6				3.1	0.8	0.8			
9	820	3.1	3.1	1.6	6.2	1.6	0.4	1.6	1.6	0.8	1.6	0.8	0.4
10	580	3.1	3.1	1.6	6.2	3.1	1.6	1.6	1.6	0.8	1.6	0.8	0.4
11	740				12.5	3.1	0.8				1.6	0.8	0.4
	MEDIAN	6.2	3.1	1.6	6.2	3.1	0.8	1.6	1.6	0.8	1.6	0.8	0.4

cilli in intraperitoneal agar discs in ten cats receiving chlortetracycline and chloramphenicol have been shown graphically in Figure 1. The curves of the serum concentrations can be divided into two groups with respect to the duration of antityphoidal effect. Concentrations of either drug of 6 to 100 micrograms per ml. for 24 hours in the serum of the animals completely inhibited the formation of grossly visible colonies of *S. typhosa* in the agar discs for 48 hours. The corresponding concentrations of drug in the agar discs were 2.6 to 20 micrograms per ml. for chlortetracycline and less than 6.2 to 50 micrograms per ml. for chloramphenicol. In order to produce these high serum concentrations, intravenous doses of chlortetracycline of 50 to 75 mg. per Kg. or oral doses of chloramphenicol of 100 to 200 mg. per Kg. were required.

Although no visible colonies were detected in the discs removed from these cats at 48 hours, 20 to 30 large colonies of typhoid bacilli regularly appeared in each disc after a further period of incubation for 24 hours in broth. Equal numbers of large colonies appeared within 12 to 24 hours in control discs which were incubated in cats which had received no antimicrobial drug and in infusion broth *in vitro*. No colonies were observed in control discs incubated in saline for 48 hours.

Lower concentrations of the two drugs were less effective in suppressing the growth of *S. typhosa*. Thus, serum concentrations of 0.8 to 25 micrograms per ml. of either drug maintained for 24 hours were effective in preventing the appearance of visible colonies at 24 hours, but after 48 hours 20 to 30 large colonies were present in the depths of the agar. The corresponding concentration of chlortetracycline in the agar discs was 0.6 to 5 micrograms, while chloramphenicol could not be measured. These serum and agar concentrations followed doses of chlortetracycline of 100 mg. per Kg. orally or 10 to 20 mg. per Kg. intravenously and doses of chloramphenicol of 25 to 50 mg. per Kg. orally.

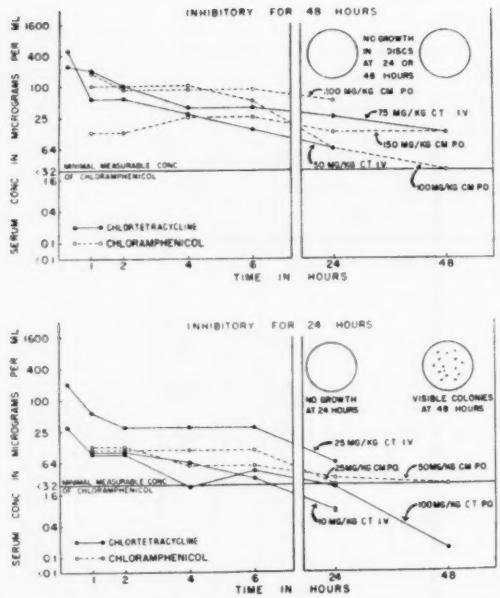

FIG. 1. EFFECT OF CHLORTETRACYCLINE AND CHLORAMPHENICOL IN SUPPRESSING S. TYPHOSA IN INTRAPERITONEAL AGAR DISCS IN CATS

DISCUSSION

The results of the present investigation indicate that one explanation for the difference of chemotherapeutic effect between chlortetracycline and chloramphenicol in typhoid fever may be a simple difference in the drug-host relationships of the two compounds. Equal serum concentrations of the two drugs exhibited equal effect in suppressing the growth of typhoid bacilli *in vivo,* but the doses of chlortetracycline which were required to produce these concentrations were much higher than those of chloramphenicol. Intravenous doses of chlortetracycline of 50 and 75 mg. per Kg., for example, are equivalent to oral doses of 250 and 375 mg. per Kg. and are very much larger than the conventional doses used in clinical practice. In a previous study in humans it was shown that oral doses of chloramphenicol of 50 to 100 mg. per Kg. yielded serum concentrations which were well in excess of the minimum inhibitory concentrations of this drug for *S. typhosa,* whereas the same doses of chlortetracycline were inadequate in this respect (12).

The minimum inhibitory concentrations of the two drugs *in vitro* were not greatly different from each other and were of the same magnitude as those found by other investigators (5, 6). Although the inhibitory concentrations of chlortetracycline in the serial dilution method were two to four times greater than those of chloramphenicol on a weight-volume basis, a two-fold difference by this method is within the normal range of error. Moreover, no correction was made for the deterioration of chlortetracycline which may occur at incubator temperatures and which would have the effect of increasing the concentration of this drug required for suppression of bacterial growth (13). Chloramphenicol, on the other hand, has been found to be markedly stable to heat and does not lose potency under these conditions (14). The molar concentrations of the two compounds required to inhibit *S. typhosa* are more nearly equal than the weight-volume concentrations, for the molecular weight of chlortetracycline is almost twice that of chloramphenicol, 508 and 310, respectively (15, 16). Serum concentrations two or more times the minimum inhibitory concentrations *in vitro* were required for both drugs to prevent the growth of typhoid bacilli *in vivo,* and when the serum concentrations fell below these values bacterial colonies became visible in the depths of the agar. The bacteriostatic action of the two drugs as opposed to a sterilizing action was confirmed both *in vitro* and *in vivo.*

Certain clinical data have also supported the notion that the irregular effect of chlortetracycline in typhoid may be due, at least in part, to a drug-host factor and that this may be overcome under certain circumstances. In a study by other investigators (10) two patients with typhoid who received very large doses of chlortetracycline, 350 and 500 mg. per Kg., orally, showed a very prompt recovery within 72 hours, and 12 other patients treated with large doses improved more rapidly than untreated controls. In general, however, in the doses ordinarily used in clinical practice chlortetracycline has been relatively ineffective in typhoid fever while chloramphenicol has had a regular and predictable beneficial effect (10, 17). This has been in contrast to the high order of therapeutic effectiveness of both drugs in other predominantly intracellular infections such as acute brucellosis and rickettsial infections.

The conditions under which typhoid bacilli exist in agar discs in animals are obviously not the same

as those in the lesions of typhoid fever. Since the agar was impervious to inflammatory cells of the host in these experiments (11), the bacilli were not situated intracellularly. It was impossible, therefore, by the present method to assay other variables such as intracellular parasitism which conceivably might influence the antityphoidal activity of chlortetracycline and chloramphenicol *in vivo*. In studies with other microorganisms, however, the tetracycline drugs have been shown to be active in the same concentrations on bacteria situated within monocytes as on bacteria in the extracellular environment (18, 19). Whether the other types of host cell parasitized by typhoid bacilli (*e.g.*, plasma cells) (20) are as easily penetrated by the tetracyclines as the monocyte, has not yet been established.

SUMMARY

A study has been made of the comparative antityphoidal activities of chlortetracycline and chloramphenicol to explain the difference of therapeutic effect of these two compounds in typhoid fever. The *in vitro* minimum inhibitory concentrations of the two drugs for *S. typhosa* were determined in parallel by the conventional serial dilution method. The *in vivo* studies were carried out in cats, and the serum concentrations after various oral and intravenous doses of the two drugs were correlated with the suppression of growth of typhoid bacilli encased in triple layered agar discs which were inserted into the peritoneal cavities of the animals. In this way the bacilli were found to be subsisting directly on nutriments supplied by the host in a state more closely approximating true parasitism than is possible *in vitro*. The agar discs were readily penetrated by both drugs from the extracellular fluid of the peritoneal cavity.

Essentially equivalent concentrations of the two compounds exerted comparable bacteriostatic effect on typhoid bacilli both *in vitro* and *in vivo*. The doses of chlortetracycline required to provide concentrations in the extracellular fluid which were inhibitory for *S. typhosa* were found to be significantly greater than those of chloramphenicol and were greater than those doses ordinarily employed in clinical practice. The results of the study indicate, therefore, that one explanation for the difference in the therapeutic activities of chlortetracycline and chloramphenicol in typhoid fever may be the difference in the drug-host, rather than the drug-parasite, relationships of the two compounds. This drug-host difference may be a critical factor in the chemotherapy of typhoid fever in contrast to the treatment of other susceptible intracellular infections in which a more uniform response to these compounds has been noted.

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STUDIES OF DIPHTHERIA ANTITOXIN IN RHEUMATIC FEVER SUBJECTS: ANALYSIS OF REACTIONS TO THE SCHICK TEST AND OF ANTITOXIN RESPONSES FOLLOWING HYPERIMMUNIZATION WITH DIPHTHERIA TOXOID

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(Submitted for publication November 16, 1953; accepted January 6, 1954)

The importance of group A hemolytic streptococcal infections in the pathogenesis of rheumatic fever is widely recognized, but little is known of the mechanism by which the streptococcal infection gives rise to the disease. The concept that an antigen-antibody interaction may be involved in the origin of the pathologic processes has received increasing attention, and recent antibody studies (1, 2) provide suggestive evidence in support of this point of view. For example, studies in several laboratories dealing with a variety of streptococcal antigens have demonstrated that the mean antibody response of a group of rheumatic fever patients to these antigens is greater than that of a comparable group of patients with uncomplicated streptococcal disease (3–6). In view of these findings, it is of importance to determine whether the hyperreactivity of rheumatic subjects is limited to antigens derived from the streptococcus or is a reflection of a fundamental difference which would be demonstrable in an enhanced response to a variety of different antigens.

Because it has been impossible in natural infections with the hemolytic streptococcus to obtain accurate quantitative information concerning the antigenic stimulus, one of the possible explanations of the relative enhancement of the immune response in rheumatic subjects is that these individuals may have been exposed to comparatively greater quantities of antigen. At the present time it is difficult to approach this problem experimentally using streptococcal antigens, but other antigens are available, some in highly purified form, for use in a comparative study of the antibody response in rheumatic and control subjects. By this approach it should be possible to determine whether the exaggerated response to an antigenic stimulus is a general characteristic of rheumatic subjects. However, a negative result would not invalidate the hypothesis that rheumatic fever is related to hypersensitivity to the hemolytic streptococcus or its products. On the other hand, a discrepancy in the responses of rheumatic and control individuals would support the thesis that rheumatic subjects possess an innate difference in the antibody forming mechanism.

In the present study, diphtheria toxoid was selected as the immunizing antigen because it was considered to possess a number of advantages over other possible materials. It is available as a highly purified substance, and sensitive procedures have been devised for the measurement of antitoxin. Furthermore, quantitative studies have shown that highly purified diphtheria toxoid and human antitoxin behave as a single antigen-antibody system. In addition, the use of this system makes it possible to obtain qualitative as well as quantitative data concerning antibody formation, since techniques are available to study both precipitating and nonprecipitating antitoxin (7–9).

The investigations to be reported are based upon skin reactions observed in 245 Schick tested rheumatic and control subjects and upon the antitoxin responses occurring in 121 of these individuals following a single booster dose of purified diphtheria toxoid.

MATERIALS AND METHODS

Clinical material. The total study group consisted of 245 individuals; 132 members gave a history of rheumatic fever or rheumatic heart disease and this group was comprised of (a) 104 adult males ranging from 18

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to 26 years of age, and (b) 28 male and female children ranging from 9 to 15 years of age. The older subjects were United States Air Force personnel stationed at the Fort Francis E. Warren Air Force Base, Wyoming.[2] The younger subjects were patients at Irvington House, Irvington-on-Hudson, New York, a convalescent rheumatic fever home.[3] Fifteen of the former and fifteen of the latter group gave a definite or suggestive history of more than one previous rheumatic fever attack. Many had had repeated demonstrable throat infections associated with the presence of group A hemolytic streptococci. However, at the time this study began the rheumatic subjects with few exceptions showed no clinical or laboratory evidence of rheumatic activity. Most of the rheumatic children had some evidence of rheumatic valvular heart disease, as did a lesser proportion of the rheumatic adults. Treatment in one form or another had been given to all individuals during the period of activity, and included administration of corticotrophin, cortisone, aspirin and hydroxyphenyl-cinchoninic acid, or a combination of these. However, the time between treatment and the administration of diphtheria toxoid ranged from 4 to 18 months in the adult group and from 1 to 11 months in the younger age group. Each of the two groups of rheumatic patients was compared with a group of control individuals of the same average age with no history of rheumatic fever or chorea and no evidence of rheumatic valvular heart disease.

Skin testing. Highly purified diphtheria toxin diluted in buffered[4] heated human serum albumin to 0.2 guinea pig MLD (0.004 Lf) per cc. was used in the Shick test[5] which was performed on all subjects in this study. Highly purified toxoid containing 0.08 Lf per cc. was used as the control. The volume injected intradermally was 0.1 cc. Earlier work (8) has shown a correlation between immediate skin reactivity of the wheal and erythema type to Schick toxoid and the presence of non-precipitating skin sensitizing antitoxin; therefore, skin tests in rheumatic and control groups were evaluated, together with other tests to be described, as an indication of possible differences in antibody reactivity. Immediate reactions were read at the Schick control site 15 to 30 minutes after performing the Schick test. The diameter of the area of wheal and of erythema were both measured and the presence of pseudopodia, pruritis, and color intensity noted. On the basis of these criteria, reactions were rated as negative, ±, +, ++, +++, ++++ (8). In the adult groups additional skin sites were injected with 0.1 cc. buffered human albumin (Schick diluent) as a control and equally strong immediate reactions against both toxoid and albumin diluent were arbitrarily classified as negative. The Schick test was read at 48 hours.

Immunization. Immunization was carried out in 121 individuals who were Schick negative and who did not show a delayed reaction to Schick control toxoid at 48 hours. After the Schick test was read, a single injection of purified diphtheria toxoid was given subcutaneously. The dose was 37.5 Lf fluid toxoid or 200 Lf of purified toxoid absorbed on alumina cream[6] (11). Forty-four adult male Schick negative rheumatic subjects and an equal number of non-rheumatic male controls received 37.5 Lf fluid toxoid (1 cc.). Twenty-two Schick negative rheumatic children (17 male and 5 female) and 11 male controls received 200 Lf alum toxoid (1 cc.). Local reactions subsequent to immunization were mild or non-existent except in three instances where a febrile response was accompanied by considerable local swelling and discomfort.

Pre-immunization bleedings were taken at the time the Schick tests were given. Following the booster dose of toxoid given 48 hours later, post-immunization bleedings were drawn between the first and second, and second and third weeks, usually the ninth and nineteenth days, and in certain cases at intervals thereafter. Serum was removed using sterile precautions and stored in small glass tubes at 4° C. until used.

Antibody determinations. The *in vivo* antitoxin titer was determined using the rabbit intracutaneous test, following the technic of Fraser (12). Unknown specimens were compared with a simultaneous titration carried out with standard antitoxin obtained from the National Institutes of Health. Dilutions were spaced in such a manner that the titrations were accurate to within 15 to 20 per cent.

Tests for specific precipitation of antitoxin with toxin were carried out by a capillary tube precipitation method (13). A single concentration of serum containing an appropriate amount of antitoxin was mixed with varying amounts of toxin. On the basis of titers obtained using the rabbit skin test, sera of sufficiently high titer were diluted to contain about 30 units per cc. Other specimens containing between 10 and 30 units per cc. were used undiluted. The amount of toxin varied from 5 Lf to 40 Lf per cc. The purified toxin used contained 2480 Lf per cc. and 1 Lf was equivalent to 0.46 μg. specifically pre-

[2] It is a pleasure to acknowledge assistance provided by Dr. Charles Rammelkamp, Director of the Streptococcal Disease Laboratory, Fort Francis E. Warren Air Force Base, Wyoming, for his many courtesies and for permission to use the facilities of his laboratories during various phases of this study. The staff of the Streptococcal Disease Laboratory assisted in the immunization of the adult subjects.

[3] The authors wish to thank Dr. Gene Stollerman, Medical Director of Irvington House, Irvington-on-Hudson, New York, for providing facilities used at the time subjects of the younger age group were given booster toxoid. Staff members of Irvington House assisted in the immunization of these subjects.

[4] Schick toxin and control were diluted in Glenny's borate buffer containing 0.4 per cent of heated human serum albumin and 0.02 per cent merthiolate (10).

[5] The authors are grateful to Dr. J. A. McComb and Mr. L. Levine of the Massachusetts Antitoxin Laboratory, Jamaica Plains, for this material.

[6] The alum toxoid used in this study was kindly provided by Dr. John Osborne, New York University College of Medicine, New York, New York.

cipitable Nitrogen.[7] The approximate point of equivalence was taken as the tube in which maximal precipitation resulted following two hours of incubation at 37° C. and overnight incubation at 4° C. Quantitative precipitin titers were performed in the case of some sera by methods described elsewhere (7) and *in vivo/in vitro* ratios were determined in these instances. This ratio indicates the proportion of total antitoxin that is precipitable and thus provides an index of the amount of non-precipitating antibody.

Criteria used to determine the presence of human non-precipitating antitoxin have been described in detail in another publication (9): the criteria used in this study included immediate skin reactivity to Schick toxoid, the relative ability of antitoxin containing sera to form specific precipitates in the presence of purified toxin or toxoid, and the ability to fix guinea pig complement. Individuals showing immediate skin reactivity to Schick toxoid possess non-precipitating, skin sensitizing diphtheria antitoxin. Sera containing this type of antitoxin demonstrate poor or no fixation of guinea pig complement in contrast to precipitating antitoxin which is usually able to fix relatively large amounts of complement. The technique employed in comparing the complement fixing ability of various antitoxin-containing sera has been described elsewhere (9). Evaluation of rheumatic and control sera in regard to the presence of non-precipitating antitoxin was made on the basis of whether more or less than one unit of antitoxin in sera containing 10 units or more per cc. was capable of binding two units of guinea pig complement. Past experience has shown this to be a convenient level of demarcation in judging relative amounts of precipitating and non-precipitating antitoxin in a given serum.

Because it was thought of interest to determine the possible non-specific anamnestic rise of antibodies to a product of the streptococcus following administration of diphtheria toxoid, antistreptolysin O determinations were performed on pre- and post-immunization sera of the young-age-group rheumatic and control subjects who received alum toxoid. The method used was that described by Todd as modified by Hodge and Swift (14).

RESULTS

Reactions to the Schick test

Table Ia shows the results of the immediate wheal and erythema reactions which were produced following the injection of Schick test (toxin) and control (toxoid) materials in 195 adult rheumatic and control subjects. Of 104 rheumatic subjects, 45 or 43.3 per cent showed positive immediate reactions, and 59 or 56.7 per cent showed negative reactions to Schick toxoid. A slightly higher incidence of positives (52 or 57.1 per cent) occurred in 91 control subjects. However, most of these immediate reactions were not severe, and 75.5 per cent of the rheumatic subjects and 77 per cent of the controls were graded as ± or 1 +. Only 11 of 45 rheumatic subjects (24 per cent) and 12 of 52 controls (23 per cent) demonstrated reactions of 2 + or greater. Immediate wheal and erythema reactions were not analyzed in rheumatic subjects comprising the younger age category. Analysis of delayed skin reactions to Schick toxin and toxoid (Table Ib) in the older subjects showed that in the rheumatic group 39 were Schick positive (37

TABLE I

Skin reactions to Schick toxin and toxoid in 104 adult rheumatic and 91 adult control subjects

	Rheumatic	Control
a. Immediate wheal and erythema reactions to Schick toxoid		
Positive	45 (43.3%)	52 (57.1%)
±	16	8
1+	18	32
2+	4	8
3+	5	3
4+	2	1
Negative	59 (56.7%)	39 (42.9%)
b. Schick test reactions		
Schick positive	39 (37.5%)	42 (46.2%)
Schick negative	53 (51.0%)	35 (38.5%)
Pseudo reaction	12 (11.5%)	14 (15.4%)

TABLE II

Comparison of immediate wheal and erythema reactions to Schick toxoid before and after hyperimmunization of Schick negative adult rheumatic and control subjects

		Rheumatic	Control
Before immunization	Positive	19 (45.2%)	18 (54.5%)
	±	7	4
	1+	7	9
	2+	1	3
	3+	2	1
	4+	2	1
	Negative	23 (54.8%)	15 (45.5%)
		42	33
After immunization	Positive	32 (78%)	29 (87.9%)
	±	1	1
	1+	8	13
	2+	9	7
	3+	7	5
	4+	7	3
	Negative	9 (22%)	4 (12.1%)
		41	33

[7] We wish to thank Dr. A. M. Pappenheimer, Jr. who made available the highly purified diphtheria toxoid used for the precipitation tests.

per cent), 53 Schick negative (51 per cent) and 12 pseudoreactors (11 per cent). In the control group, 42 were Schick positive (46 per cent), 35 Schick negative (38 per cent) and 14 pseudoreactors (15 per cent).

The role of hyperimmunization with toxoid in altering immediate skin reactivity to Schick toxoid was demonstrated in 75 Schick negative adult subjects, 42 of them rheumatic and 33 control. Table II indicates that there was a large increase in the percentage of subjects showing immediate skin reactions in both the rheumatic and control groups, although there was no significant numerical difference between the groups. Similar data are not available for Schick tested subjects in the younger age category.

It was concluded from these results that rheumatic and control subjects reacted similarly to intradermal Schick toxin and toxoid.

Amounts of antitoxin produced following hyperimmunization with diphtheria toxoid

The titers determined by rabbit skin test on two different dates following immunization were compared in the rheumatic and control groups and showed that the mean antitoxin responses were the same, as shown in Table III. It was also demonstrated that the titers varied over a wide range in both groups at nine and nineteen days following immunization.

A comparison of the amounts of antitoxin formed showed that a majority of the post-immunization responses were at levels of 50 units per cc. or less, comprising 78 per cent and 66 per cent at nine and nineteen days in rheumatic subjects, and 68 per cent and 67 per cent at corresponding times in the controls. Only 11 per cent of persons in both groups formed more than 100 units per cc. at nine days, and 24 per cent of rheumatic and 19 per cent of control subjects developed titers higher than 100 units per cc. at nineteen days.

TABLE III

Comparison of antitoxin titers in 44 adult rheumatic and 44 adult control subjects hyperimmunized with diphtheria toxoid

	9 Days following immunization		19 Days following immunization	
	Rheumatic	Control	Rheumatic	Control
Lowest titer (*units/cc.*)	1	1	1	1
Highest titer (*units/cc.*)	400	140	300	250
Mean titer (*units/cc.*)	44.3	41.4	57.6	55.1

TABLE IV

Comparison of antitoxin titers in 22 rheumatic and 11 control children hyperimmunized with diphtheria toxoid

	9 Days following immunization		19 Days following immunization	
	Rheumatic	Control	Rheumatic	Control
Lowest titer (*units/cc.*)	1	10	4	3
Highest titer (*units/cc.*)	240	440	100	320
Mean titer (*units/cc.*)	45.9	115.0	38.2	75.4

The mean titer response in young rheumatic children (Table IV) was the same as that of the adult series at nine days, but was considerably less than the mean adult response at nineteen days. As in the adult group, a wide range in titer responses was demonstrated. Greater quantities of antitoxin were formed in the young control subjects. This is a factor of doubtful significance because the small group (11 subjects) included two persons who formed particularly large amounts of antitoxin. If these two members are eliminated from the series, mean titers are obtained which approximate those of the rheumatic patients.

There seems to be no apparent difference in the post-immunization antitoxin response between subjects giving a history of a single attack of rheumatic fever and patients with a history of more than one attack. Comparison was made between maximum titers observed in 49 rheumatic children or adults who gave a history of but one attack of rheumatic fever, and in 17 subjects who gave a history of more than one rheumatic fever attack. The range of titer in the former group was from 1 to 300 units per cc. with a mean titer of 46 units per cc.; in the latter group the titer also ranged from 1 to 300 units per cc. with a mean titer of 45 units per cc.

The foregoing evidence suggests that the antitoxin response is quantitatively similar in rheumatic and control subjects.

Nature of the antitoxin produced following hyperimmunization with diphtheria toxoid

In order to determine whether rheumatic subjects possessed a superior ability to form nonprecipitating antitoxin compared with the con-

TABLE V

Complement fixation by antitoxic sera from 26 adult rheumatic and 25 adult control subjects hyperimmunized with diphtheria toxoid*

	Amount of antitoxin bound by two units of guinea pig complement		
	Under 1 unit	Over 1 unit	Totals
Rheumatic	16 (61.5%)	10 (38.5%)	26 (100%)
Control	16 (64%)	9 (36%)	25 (100%)

* All sera used in this experiment contained 10 units/cc. or more.

trols, both groups initially were compared on the basis of immediate skin reactivity to Schick toxoid. The results in 195 subjects, previously analyzed under *Reactions to the Schick test* (see Tables I and II), showed no significant differences between the groups. It has been possible to obtain additional data concerning the nature of antitoxin in a lesser number of persons whose sera were tested for antitoxin by the qualitative and quantitative precipitin tests and by the guinea pig complement fixation test. The sera used for these experiments contained 10 or more units of antitoxin per cc.

There was a marked similarity in reactions demonstrated by sera in both groups (total of 49 subjects tested) as judged by the ability to fix a standard amount of guinea pig complement (see Table V). Analogous results were also observed when the sera of 50 subjects were tested for the ability to form precipitate when mixed with an approximately equivalent amount of toxin using the qualitative technique (Table VI). In order to demonstrate possible quantitative differences, quantitative precipitin titers were carried out using sera of sixteen rheumatic patients in the younger age category. All of these sera contained 15 units per cc. or more as judged by the rabbit skin test. The *in vitro* titer was taken as the number of micrograms of antitoxic nitrogen precipitable following the addition of equivalent amounts of toxin. The *in vivo/in vitro* ratio was the figure obtained upon division of the titer as judged by the rabbit neutralization test (*in vivo*) by the *in vitro* titer. Cohn and Pappenheimer (7) found that the ratio varied from 1.0 to 1.8 in a study of 12 apparently normal subjects. The ratios calculated for the sixteen rheumatic sera studied quantitatively showed a similar range of distribution

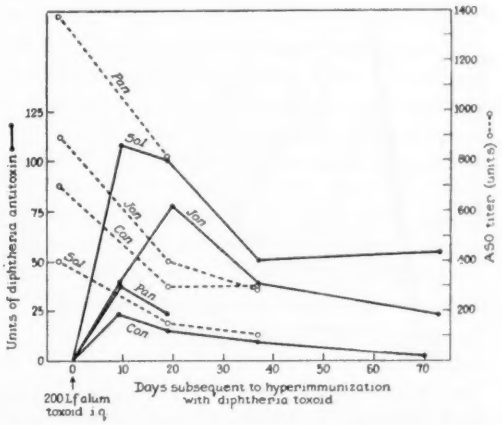

FIG. 1. ANTISTREPTOLYSIN O AND DIPHTHERIA ANTITOXIN RESPONSE IN HYPERIMMUNIZED RHEUMATIC CHILDREN WITH RECENT STREPTOCOCCAL INFECTIONS

with the exception of one subject who formed 40 units per cc. of antitoxin as judged by the rabbit skin test in the absence of any precipitating antitoxin.

The above tests revealed no apparent significant differences between rheumatic and control groups in regard to the nature of the antitoxin formed subsequent to booster toxoid.

The course of immune responses subsequent to booster toxoid

In many instances it was not possible to obtain follow-up serological studies beyond 30 to 40 days. However, in a small number of persons sera were studied up to 80 days following immunization. The antitoxin responses of four rheumatic and five control subjects in the 9 to 15 year age group who received alum toxoid are shown in Figures 1 and 2. Comparison of these data in both groups shows that the period of time during which the

TABLE VI

Incidence of precipitating and non-precipitating antitoxic sera in 25 adult rheumatic and 25 control subjects hyperimmunized with diphtheria toxoid*

	Precipitating	Non-precipitating	Totals
Rheumatic	20 (80%)	5 (20%)	25 (100%)
Control	18 (72%)	7 (28%)	25 (100%)

* All sera used in this experiment contained 10 units/cc. or more.

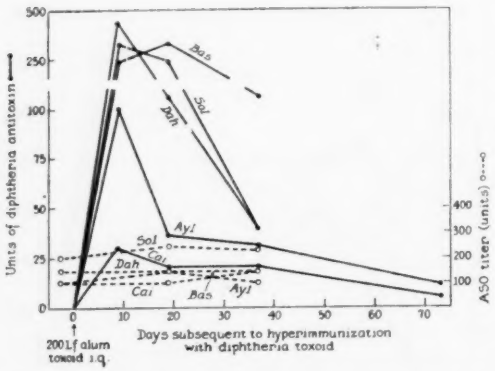

FIG. 2. ANTISTREPTOLYSIN O AND DIPHTHERIA ANTITOXIN RESPONSE IN HYPERIMMUNIZED NON-RHEUMATIC CHILDREN

largest amounts of antitoxin were produced was in the interval between 10 and 20 days after immunization. The antitoxin titer decreased markedly during the next twenty days and much more slowly thereafter. Exceptions to this type of response occurred, as in the sera from rheumatic patients Rod and Dav (Figure 3). Although the follow-up period in the numerically greater adult series is short, the rheumatic and control groups are similar in regard to the course of antitoxin response.

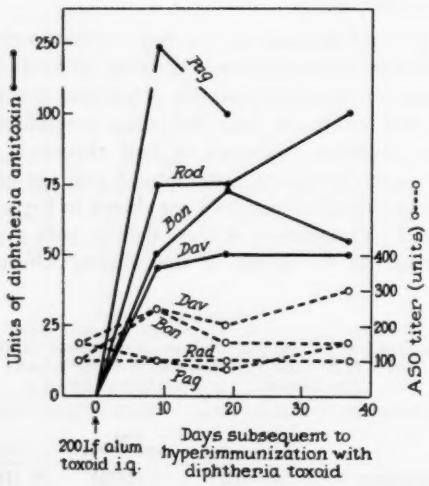

FIG. 3. ANTISTREPTOLYSIN O AND DIPHTHERIA ANTITOXIN RESPONSE IN HYPERIMMUNIZED RHEUMATIC CHILDREN WITH REMOTE STREPTOCOCCAL INFECTIONS

Behavior of antistreptolysin O titer before and after immunization with diphtheria toxoid

Sera from the 33 subjects (ages 9 to 15) who received alum toxoid were tested for antistreptolysin O levels before and after booster toxoid. Because it was thought that rheumatic subjects with very recent attacks might prove refractory to an otherwise demonstrable immune stimulus, it was felt advisable to divide the rheumatic subjects into two categories: (a) 11 patients in whom rheumatic fever had been quiescent for less than six months; (b) 11 patients in whom rheumatic fever had been quiescent longer than six months. Patients in the first group almost without exception showed significant decreases in the antistreptolysin O titer during the period of this study, suggesting relatively recent exposure to group A hemolytic streptococci. Individual titers ranged from 150 to 1400 units prior to immunization with diphtheria toxoid and from 100 to 800 units following booster toxoid, with mean values of 605 and 250 units, respectively. Subjects in group (b) did not show significant variations in the antistreptolysin O titer during the study, suggesting more remote contact with group A hemolytic streptococci. In this series, titers ranged from 100 to 300 units before and after toxoid with mean values of 142 and 182 units, respectively. In neither group was there demonstrable evidence of a significant increase in the antistreptolysin O titer following the injection of diphtheria toxoid during which time a rise and fall in the titer of diphtheria antitoxin occurred. This is shown in Figures 1 and 3. Similar findings were observed in the non-rheumatic control group (Figure 2).

DISCUSSION

The data indicate that rheumatic and control subjects react similarly following a single booster dose of diphtheria toxoid and that the number of previous attacks of rheumatic fever appears to have no bearing upon the ability to form diphtheria antitoxin. In most individuals there occurs a rise in antitoxin titer, oftentimes to high levels, within a matter of days following a booster dose of diphtheria toxoid. A wide spread occurred in both groups with respect to the amounts of antitoxin formed, although the mean values were essentially the same in all groups with the exception

of the numerically small group of control children. These results are in general accordance with the findings of Quinn, Seastone, and Dickie (15) for pneumococcus type I polysaccharide and of Miller, Kibrick, and Massell (16) for influenza vaccine. Similarly Miller, Kibrick, and Massell (16) found considerable overlapping of antibody response to typhoid antigen in rheumatic and control groups. However, they found a small but significant increase in the mean titer of rheumatic subjects as compared with controls. None of the foregoing evidence can preclude the possibility of a limited form of sensitivity restricted to antigens of the group A hemolytic streptococcus or its products.

The present study is concerned only with the secondary variety of immune response. All immunized persons were Schick negative and, therefore, possessed a detectable amount of circulating antitoxin prior to booster dose of toxoid. In this respect the present study differs from that of Miller, Kibrick, and Massell (16) in which typhoid vaccine was used. All individuals used in their studies presumably received primary immunization. However, the demonstration of a similarity of immune response in rheumatics and controls to some antigens (15, 16) given as primary, and others as secondary immunization suggests that antigenic conditioning under the present set of experimental circumstances is not one of crucial importance. In view of the possibility that repeated streptococcal infections may be of importance in the pathogenesis of rheumatic fever (17), the secondary type of response may be of greater significance in immunological studies related to the rheumatic state.

Because of the possibility that an unusual type of antibody may play a role in rheumatic fever, it was thought of importance in the present work to determine whether differences occur between rheumatic and non-rheumatic subjects in the type of diphtheria antitoxin produced. Earlier studies (8) have shown that human subjects immunized with purified diphtheria toxoid form either precipitating antitoxin, non-precipitating antitoxin, or a mixture of both. The present investigations confirm the original studies, and in addition, show no significant numerical differences between rheumatic and control groups in the ability to form one or both varieties of antitoxin as measured by the immediate skin test, specific precipitability, the *in vivo/in vitro* ratio, and fixation of guinea pig complement.[8] The possibility that rheumatic patients more readily produce a non-precipitating form of antibody, or a group of such antibodies to certain products of the group A hemolytic streptococcus cannot be ruled out on the basis of the studies described, since an unrelated antigen was used. However, a generalized tendency to this kind of immune response in rheumatic individuals is not suggested by our results.

There is some evidence which indicates that so-called anamnestic antibody responses occur following exposure to, or stimulation with, unrelated materials (18–20), but there is likewise a considerable body of experimental data (21–23) to show that anamnestic responses occur only subsequent to stimulation with antigenically related substances. In the present study, the absence of rises in antistreptolysin O during the period of maximum diphtheria antitoxin response is in accord with the view that non-specific antibody responses do not occur.

SUMMARY

The present study was based upon analysis of skin reactions to Schick toxin and toxoid in 245 rheumatic and control subjects, and upon antitoxin responses in 121 of these individuals following a single booster dose of purified diphtheria toxoid.

The amounts of antitoxin produced in rheumatic and control subjects subsequent to a booster dose of diphtheria toxoid were similar with regard to the range of titer and the mean titer. The course of antitoxin responses in rheumatic patients studied up to 80 days was similar in most instances to that of control subjects.

[8] On the basis of current investigations in this laboratory, it is probable that non-precipitating antitoxin occurs in a form which does not sensitize human skin. This possibility was raised with regard to the original studies (8) because a few sera containing non-precipitating antitoxin possessed relatively high antitoxin titers in the presence of only moderate skin reactivity to toxoid. In the present investigation, several individuals in both rheumatic and control groups who demonstrated no reactivity to Schick toxoid possessed antitoxin which was not precipitable by toxoid. Further related studies using additional immunological techniques are now in progress. The above finding would seem to have no bearing on the present studies, since there was no significant difference in the number of precipitin formers (Table VI) or of immediate skin reactors (Tables I and II) between the rheumatic and control groups.

There was no demonstrable difference in the post-immunization antitoxin response between subjects with a history of one rheumatic fever attack and patients with a history of more than one attack.

Comparison of the relative ability of rheumatic and control subjects to form non-precipitating antitoxin was made on the basis of immediate wheal and erythema reactions to Schick toxoid, specific precipitability with toxoid, analysis of the *in vivo/in vitro* ratio and guinea pig complement fixation. The results of these tests in both groups showed no significant differences.

The above data indicate that, qualitatively and quantitatively, antitoxin responses to diphtheria toxoid are approximately the same in rheumatic and non-rheumatic individuals.

Antistreptolysin O titers, determined in rheumatic and control children during the period of maximal diphtheria antitoxin formation, showed no consistent rises in either group.

ACKNOWLEDGMENT

We wish to thank Mrs. Katia Jacobs and Miss Betsy House for technical assistance during certain portions of this study.

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THE CARDIAC OUTPUT IN PATIENTS WITH CIRRHOSIS OF THE LIVER AND TENSE ASCITES WITH OBSERVATIONS ON THE EFFECT OF PARACENTESIS

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(Submitted for publication August 25, 1953; accepted January 27, 1954)

Previous studies in this laboratory (1) have indicated that the cardiac output may be elevated in patients with Laënnec's cirrhosis. The question arose as to what role distention of the abdomen by ascites might play in determining the level of systemic blood flow. In the previously reported patients, elevation of cardiac output did not appear to be related to the presence or degree of ascites, but this aspect of the problem required further investigation.

Few studies of the effect of ascites on cardiac output in man have come to our attention. Resnik, Friedman, and Harrison (2) estimated cardiac output in patients with congestive heart failure by means of the acetylene method and noted slight decreases during the accumulation of ascites in four patients. Six hours after paracentesis, performed in one subject, the cardiac output fell from 2.1 liters per min. to 1.6 liters per min.; on the following day it was 1.8 liters per min. Inasmuch as the oxygen consumption decreased proportionately, the arterio-venous oxygen difference remained essentially unchanged. Sherlock (3) studied two patients with tense ascites by means of cardiac catheterization and found that intraperitoneal, intrapleural and right auricular pressures rose after intraperitoneal infusion of saline solution and fell after paracentesis, but the cardiac output remained unaffected.

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CASE MATERIAL AND PLAN OF STUDY

Thirteen hospitalized patients with Laënnec's cirrhosis and tense abdominal ascites were studied. All gave a history of excessive consumption of alcoholic beverages. History, physical examination, fluoroscopy of the chest, and electrocardiogram were obtained in all patients and revealed no evidence of organic heart or lung disease. Studies were made in the fasting state, at rest in recum-

TABLE I

Pertinent data of patients with Laënnec's cirrhosis not previously reported

Case No.	Age and sex	Weight* *lbs.*	Body† surface area *m²*	Cardiac output *L./min.*	O_2 cons. *cc. STPD*	Heart rate
			Cirrhosis with tense ascites			
23	44 M	160	1.85	9.14	358	122
24	41 M	257	2.34	10.67	377	106
25	51 F	170	1.84	9.70, 11.33 } 10.52‡	327, 329 } 328‡	120
26	46 M	178	1.95	8.45	240	87
			Cirrhosis without ascites or edema			
27	42 M	185	2.00	9.16	371	74
28	41 M	168	1.95	15.05	320	95
29	52 M	158	1.81	6.74	296	70
30	44 F	128	1.63	5.93	232	75
31	57 M	176	1.90	6.70	267	92
32	39 F	107	1.50	5.39, 5.39 } 5.39‡	296, 304 } 300‡	110

* Actual weight ("wet").
† On basis of actual weight.
‡ Mean of two determinations.

TABLE II

Circulatory measurements before and after paracentesis

Study No.	Case No.	Age	Sex	Body* surface area	Weight	Ascites 0–4+	Periph. edema 0–4+	Time of study	Ascitic fluid removed	Heart rate	Resp. rate	Minute vent. STPD	Oxygen consump. STPD	Cardiac output	Stroke volume	Brachial arterial pressure		Calculated peripheral resistance	Hematocrit
																Phasic	Mean		
				m²	*lb.*				*L.*				*cc./min.*	*L./min.*	*cc.*	*mm. Hg*		*Dynes-cm.⁻⁵ sec.*	*%*
1.	22	62	M	1.91	216	4+	2+	24 h A†		93	—	—	—	6.64	71	160/95	117	1410	31
					186	0	0	24 h P	10+	94	—	—	—	6.85	73	—	—	—	33
2.	33	46	M	1.65	149	4+	1+	2 h A		98	18	7.06	246	7.82	80	124/78	88	900	35
					121	0	1+	50 min. P	13	105	20	6.91	212	6.72	60	110/21	78	930	30
3.	34	59	M	1.68	152	4+	0	48 h A		94	23	7.39	332	10.7	114	111/69	88	658	40
					134	0	0	2 h P	10	88	—	—	—	17.4	196	134/88	99	456	37
					134	0	0	3 h P		86	17	5.13	270	8.55	100	—	—	—	38
4.	35	59	M	1.73	175	4+	1+	90 min. A		81	—	6.68	241	6.86	85	134/74	92	1070	24
					161	1+	1+	30 min. P	7.5	74	—	—	—	7.25	98	122/62	82	905	24
					161	1+	1+	50 min. P		74	—	5.90	235	7.25	98	—	—	—	23
					161	1+	0	7 d P		74	—	6.16	234	7.60	100	130/66	91	960	21
5. a	36	41	M	1.76	185	3+	1+	3 mos. A		108	19	9.40	346	11.2	104	132/64	80	571	26
					213	4+	4+	1 h A		127	23	7.92	326	12.6	99	104/54	68	431	30
					185	0	4+	40 min. P	12	116	25	7.93	306	17.3	149	104/50	68	314	30
5. b					190	4+	2+	7 d P 1 h A		117	13	5.88	246	10.85	93	110/56	72	399	30
					156	0	2+	30 min. P	10	121	18	6.58	258	12.33	102	96/48	58	282	31
					172	2+	1+	8 d P		112	17	6.70	264	10.63	95	96/50	58	327	29

* Body surface area on basis of weight after paracentesis. † A = Before paracentesis; P = After paracentesis; xxxxxxxxxxx designates paracentesis.

TABLE III

Comparison of the cardiac output in cirrhosis with tense ascites and without ascites

Expression of blood flow	Tense ascites Mean ± S.D.	No ascites Mean ± S.D.	Difference of means / S.E. difference
Cardiac output *L./min.*	10.15 ± 4.60 (13)†	7.73 ± 2.88 (17)	$\frac{2.42}{1.42} = 1.70$
Cardiac index *L./min./M²* *	5.26 ± 1.99 (13)	4.36 ± 1.30 (17)	$\frac{0.90}{0.63} = 1.42$
Cardiac output *cc./cm. height*	60.3 ± 26.0 (13)	45.0 ± 15.4 (17)	$\frac{15.3}{8.13} = 1.88$
Cardiac output, *L./min.* × *100* / cc. oxygen consumption/min.	3.27 ± 0.51 (11)	2.81 ± 0.89 (15)	$\frac{0.46}{0.28} = 1.64$

* Body surface estimation based on "wet" weight. Mean Cardiac Index of 24 normal subjects: 3.60 ± 0.62, L./min./M² (5).

† ( ) = Number of patients.

bency. Five of the 13 patients with tense ascites were studied on six occasions before paracentesis and again at varying intervals after paracentesis. Full hemodynamic data are not reported here on the eight patients with tense ascites who were not followed through paracentesis, since only the values of cardiac output are germane to our discussion. The complete data on four of these eight patients, however, have been reported previously (1).[3] The mean cardiac output of this group of 13 patients with tense ascites was compared to that of a group of 17 patients with Laënnec's cirrhosis without evidence of ascites and/or edema, otherwise comparable, studied in identical manner. Full data on 11 of these 17 "dry" cases have been presented previously (1).[4] Pertinent data on all patients not previously reported are presented in Tables I and II.

METHODS

The *cardiac output* was determined by the dye-injection method as described by other investigators (4) with minor modifications (1). Five milligrams of Evans blue dye were injected into an antecubital vein, and samples of arterial blood were collected from the contralateral brachial artery. This method, in our hands, yields reproducible results. Values of cardiac output in normal subjects agree with accepted standards (1, 5).

The *oxygen consumption* in general was determined over a 3-minute period by the open-circuit method, the cardiac output being determined during the second minute. Expired air was collected in a Douglas bag and the volume measured in a Tissot spirometer. The concentration of oxygen was measured by means of a Pauling oxygen analyzer.[5] The carbon dioxide was not measured. The average R.Q. correction of other investigators (6) was used for converting expired volume to inspired volume. In two instances oxygen consumption was measured over a 6-minute period by means of a closed-circuit water spirometer filled with oxygen, after the determination of the cardiac output (study No. 3, Table II).

Phasic and mean *arterial pressures* were measured directly by means of an electromanometer[6] and recorded on a direct writing oscillograph.[6]

RESULTS

I. *The cardiac output at rest*

The data not previously reported are given in Tables I and II; a comparison of the means is presented in Table III. The mean resting cardiac output in the 13 subjects with tense ascites was 10.15 liters per min., with a standard deviation of ± 4.50, the sample ranging from 6.64 liters per min. to 24.2 liters per min. Seventeen patients without ascites had a mean cardiac output of 7.73 ± 2.88 liters per min. Thus the mean cardiac output in the presence of tense ascites was greater than in the control group without ascites, but this difference in means was found not to be significant statistically, nor was there any significant difference when the comparison between the group with ascites and the group without ascites was made on the basis of body surface area, the values for the cardiac index being 5.26 ± 1.99 liters per min. per M² and 4.36 ± 1.30 liters per min. per M², respectively (Table III). The mean cardiac index of 24 nor-

[3] In the cited reference these subjects were Nos. 15, 17, 19, and 20.

[4] These subjects were Nos. 4–14 in the paper cited.

[5] A. O. Beckman, Inc., Pasadena, California.

[6] Sanborn Company, Cambridge, Massachusetts.

mal subjects studied in this laboratory under comparable conditions (5) was 3.60 liters per min. per M^2 with a standard deviation of $\pm$ 0.62 liters per min. per M^2.

The presence of ascites will increase the estimate of body surface area from height and weight, hence the cardiac index calculated on the basis of "wet" weight (as done here) is probably not comparable to the cardiac index of normal subjects or patients without fluid retention. Therefore, comparisons of cardiac output between the two groups were also made on the basis of height (cc. of blood flow per minute/height in cm.) and oxygen consumption (liters of blood flow per minute $\times$ 100/cc. oxygen consumption per minute). The mean cardiac output, irrespective of the expression used, was higher in the group of patients with tense ascites, but none of the differences were statistically significant (Table III).

Thus the cardiac output in cirrhotic subjects with tense ascites was often elevated, at times normal, but never absolutely low, and in this sample did not differ significantly from that in cirrhotic subjects without ascites.

II. *The response to paracentesis* (Table II, Figure 3)

There were five instances in which studies were performed within three hours or less after paracentesis. In three of these studies, Nos. 2, 4, and 5b, the cardiac output did not change appreciably immediately after paracentesis. In study No. 3 the cardiac output rose from 10.7 liters per min. to 17.4 liters per min. two hours after paracentesis, at which time the oxygen consumption unfortunately was not measured. One hour later the cardiac output had fallen below the original level to 8.5 liters per min., in keeping with a comparable fall in oxygen consumption. In study No. 5a the cardiac output rose from 12.6 liters per min. to 17.3 liters per min. without appreciable change in oxygen consumption.

In four instances the cardiac output was measured one or more days after paracentesis. At no time was it found to differ appreciably from that existing prior to paracentesis. In two of these four studies, Nos. 5a and 5b, the individual was reaccumulating ascitic fluid as judged by an increase in weight of five pounds and six pounds, respectively.

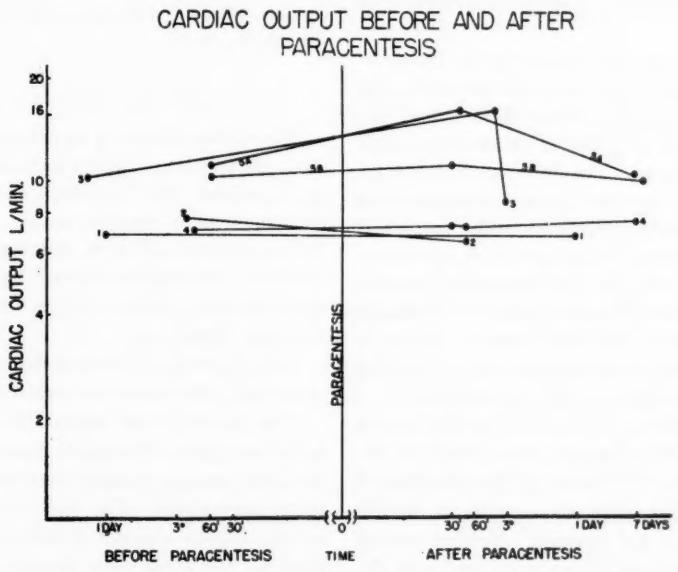

FIGURE 1

Logarithmic scales are used on the ordinate to facilitate comparison of relative changes at different levels of cardiac output, and on the abscissa for convenience.

Changes in heart rate, blood pressure, peripheral resistance, oxygen consumption and hematocrit were somewhat variable and showed no consistent trend.

DISCUSSION

The results obtained in the new cases confirm the previous findings (1): the cardiac output is often elevated in patients with Laënnec's cirrhosis, with or without ascites. A discussion of the possible causes of the high cardiac output state was presented in a previous report to which the reader is referred (1).

The data presented fail to support the thesis that tense abdominal ascites impairs venous return to the heart and thus compromises cardiac output (7). A low cardiac output was not found in any instance and high outputs were observed in a number of patients with marked ascites. These observations were made at rest and in recumbency and do not exclude the possibility that venous return might be affected during exercise. It is also conceivable that with greater degrees of abdominal distention venous return might be impaired. However, it is our opinion that patients sicker than the present group could be studied only with great difficulty and uncertainty as to the achievement of a steady state.

Since Sherlock (3) has shown that in the presence of tense ascites right auricular pressure may be increased, the question arises whether tense ascites *per se* may be a factor in producing a high cardiac output at rest. The increase in auricular pressure, however, appears to be secondary to an increase in intrathoracic pressure which in turn is effected by an increase in intraperitoneal pressure. Inasmuch as the relative right auricular pressure (referred to as intrathoracic pressure) probably remains unchanged, increased venous return is not likely. Indeed, in no instance was a true fall in cardiac output observed after paracentesis.

With two exceptions the level of cardiac output was not appreciably changed after relief of abdominal distention. The cause of the elevation of cardiac output early after paracentesis in studies Nos. 34 and 36a is not obvious. Neither patient showed overt emotional disturbance, and both denied feeling nervous or tense. In both instances subsequent determinations showed lower values. Technical errors cannot be entirely ruled out.

Shifts in body water and extracellular fluid after paracentesis might affect the level of cardiac output. Ascitic fluid forms when forces favoring the passage of extracellular fluid across the capillary walls exceed those favoring its return. The cirrhotic subject with tense ascites is at or approaching equilibrium at a new pressure relationship. The sudden removal of ascitic fluid restores the transcapillary gradient in favor of the passage of water into the peritoneal cavity, and as a consequence extracellular fluid may become "trapped" in the peritoneal cavity (8). Plasma volume may decrease since hemoconcentration has been noted after paracentesis (9). Inasmuch as a decrease in plasma volume, under certain conditions, is thought to decrease the cardiac output (10, 11), it is of interest that the present measurements of cardiac output revealed no appreciable decrease after paracentesis. Moreover, no consistent increase in hematocrit was seen.

All patients with tense ascites had dyspnea on moderate to mild exertion and several felt dyspneic at rest. Paracentesis promptly relieved such dyspnea. From the present investigation it appears unlikely that the level of cardiac output plays an important role in the dyspnea of noncardiac patients with tense ascites, at least not in the resting state.

SUMMARY AND CONCLUSIONS

The cardiac output in 13 patients with Laënnec's cirrhosis and *tense ascites* was compared with that in 17 patients with Laënnec's cirrhosis and *no ascites* in terms of absolute values, and as related to body surface area, height, and oxygen consumption. Patients with ascites showed higher mean values than patients without ascites, but the differences were not significant.

In both groups of patients the cardiac output was often high, sometimes normal, but never low.

Five patients were studied at varying intervals before and after abdominal paracentesis. In general, the resting cardiac output remained essentially unchanged. On two occasions a rise in cardiac output was noted immediately after paracentesis, but subsequent studies showed a return to control values. In no instance did the cardiac output decrease after paracentesis.

It is concluded that the presence of tense ascites

is not an important determinant of the level of cardiac output in patients with cirrhosis at rest and in recumbency.

ACKNOWLEDGMENTS

We wish to thank Dr. Laurence B. Ellis for his encouragement and assistance, Miss Vilma Grube for her invaluable technical assistance, and Dr. Charles S. Davidson for reviewing the manuscript.

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RENAL EXCRETION OF ELECTROLYTES IN PREMATURE INFANTS DURING ADMINISTRATION OF SODIUM SALTS OF UNREABSORBED ANIONS[1]

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(Submitted for publication November 24, 1953; accepted January 13, 1954)

The intravenous administration of fluids of high concentrations of sodium salts of anions not reabsorbed by the kidney tubules results, in normal adults (1–3) and children (4), in the excretion of the anions covered principally by sodium. However, in patients with nephrosis (1, 4–6) and in normal subjects given ACTH or cortisone (2, 3), potassium replaces sodium to a variable extent in covering these anions. In the normal dog, as in man, these unreabsorbed anions are excreted covered principally by sodium. However, experimentally decreasing glomerular filtration rate (GFR) acutely in the dog results to a large extent in the replacement of sodium by potassium when sodium salts of these anions are given (7, 8).

Several of these conditions under which sodium salts of unreabsorbed anions are excreted covered principally by potassium rather than by sodium obtain in the healthy premature infant. Thus, it has been observed that the premature infant does not excrete excess sodium as rapidly as the adult (9, 10) which may account for the greater tendency of the premature infant to become edematous. In addition, per unit surface area or per gram of kidney weight, GFR is lower in the premature infant than in the adult (11). For these reasons, it seemed of interest to observe the response of the premature infant to the administration of fluids of high concentrations of sodium salts of anions not reabsorbed by the kidney tubules. The results indicate that the premature infant, like the normal adult, excretes the unreabsorbed anions covered principally by sodium.

[1] This investigation was supported in part by research grants from The National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service (A-389) and from The Playtex Park Research Institute.

PROCEDURE

Eight series of observations were made on eight premature infants ranging in age from 12 to 71 days, in weight from 1964 to 2212 Gm., and in surface area from 0.155 to 0.167 M^2. All observations were made in an air-conditioned metabolism unit in which temperature and humidity were maintained at 23° C. and 50 per cent, respectively, and technics previously described (12) were used for collecting blood and urine samples in premature infants.

The premature infants were receiving half-skimmed cow's milk formulas and were offered an additional 30 ml. of water following each of the last three feedings preceding the observations. The usual procedure was to collect urine for one or two control periods after which priming and sustaining infusions of inulin, sodium chloride, p-aminohippurate (PAH) and, in most instances, thiosulfate (S_2O_3) were given (see protocols for dosages and details of each observation). Blood samples were drawn after the control period, after an equilibration period of approximately one hour and at the completion of the observations.

Serum and urine were analyzed for inulin (13), PAH (14), S_2O_3[2] (15), sodium (16), potassium (16), chloride (17–19), bicarbonate (20), and phosphate (21); ammonium ion (22), titratable acid (titrated potentiometrically to pH 7.4), and pH were measured only in

[2] Because of the low GFR and TmPAH in the premature infant, it was not possible even at high concentrations in serum to achieve concentrations of either thiosulfate or PAH in urine comparable to those obtained in adults. For this reason, sodium salts of these two unreabsorbed anions were given together to subjects 3–7. The reported interference (23) in the measurement of thiosulfate by high concentrations of PAH in filtrates of blood was not observed. On the other hand, falsely high values of thiosulfate were found in urines containing high concentrations of PAH. This difficulty was overcome by using the indirect method for urine with the precaution of always providing an adequate excess of iodate. By employing this procedure, good recoveries of thiosulfate were obtained from urines containing concentrations of thiosulfate and PAH within the range of values encountered in these observations.

TABLE I

Protocols and data on effect of infusion of sodium salts of unreabsorbed anions on electrolyte excretion in premature infants

Observation number	Subject	Period	Elapsed time	Urine flow	Inulin clearance	Urine										Blood						
						Na	K	NH_4	TA	PAH	S_2O_3	Cl	HPO_4	HCO_3	pH	Na	K	PAH	S_2O_3	Cl	HPO_4	HCO_3
			min.	*ml./min.*	*ml./min.*	*μEq./min.*										*mEq./L.*						
			0													142.4	6.2	0		109.8	3.8	27.8
1	P. E.	1	15–83	0.10		1.4	6.0	2.1	2.1	0		3.2	6.8	0.2	5.4							
	6-24-52			Priming infusion = 4.0 ml. of 10% inulin and 2.5 ml. of 20% PAH																		
	Age: 16 days		91–306	Sustaining infusion = 0.5 Gm. inulin, 0.7 Gm. PAH and 15.4 mEq. NaCl per 100 ml., given at 0.5 ml. per min.																		
	Wt.: 2031 Gm.		159															65.4				
	SA: 0.156 M^2	2	163–253	0.16	3.3	23.7	6.0	1.7	4.2	12.6		14.9	6.0	0.1	5.5							
		3	253–303	0.27	4.0	40.6	6.9	1.7	2.1	13.7		29.7	4.7	0.4	5.5							
			306													147.0	4.6	48.4		111.8	4.4	23.6
			0													146.0	6.6	0		110.2	4.1	26.2
			15–86	0.11		1.9	7.4	2.8	2.7	0		4.9	6.9	0.3								
2	K. R.			Priming infusion = 4.0 ml. of 10% inulin and 2.5 ml. of 20% PAH																		
	7-22-52	1	89–215	Sustaining infusion = 0.5 Gm. inulin, 0.7 Gm. PAH and 15.4 mEq. NaCl per 100 ml., given at 0.5 ml. per min.																		
	Age: 71 days		134												6.0			54.8				
	Wt.: 2002 Gm.	2	136–177	0.35	6.0	48.2	17.1	2.2	2.7	22.1		35.2	8.5	3.7	5.8							
	SA: 0.155 M^2	3	177–194	0.92	7.2	122.1	19.6	2.3	1.4	24.0		89.8	12.1	13.4	6.8							
		4	194–211	0.79	7.1	100.9	15.0	2.5	1.6	23.6		79.6	9.2	11.7								
			215													141.4	5.9	58.2		112.4	4.6	26.0
		1	0–32	0.14		4.1	8.1			0	0	5.6	8.3					0			5.3	
			46–81	Priming infusion = 4.2 ml. of 10% inulin. Sustaining infusion = 0.5 Gm. inulin and 16.0 mEq. NaCl per 100 ml., given at 0.5 ml. per min.																		
3	P. A.		81–177	Priming infusion = 4.2 ml. of 10% $Na_2S_2O_3$ and 2.6 ml. of 20% PAH																		
	9–16–52			Sustaining infusion = 0.5 Gm. inulin, 0.5 Gm. $Na_2S_2O_3$ and 14.6 mEq. NaCl per 100 ml., given at 0.5 ml. per min.																		
	Age: 24 days		114															38.6	29.0		4.3	
	Wt.: 2075 Gm.	2	118–140	0.19	4.4	32.2	13.9			15.0	15.9	10.1	8.9									
	SA: 0.159 M^2	3	140–156	0.29	4.3	43.1	15.1			15.7	19.7	17.8	10.4									
		4	156–173	0.31	3.7	44.6	12.5			14.1	17.8	19.6	9.1									
			177															48.7	27.3		3.8	
		1	0–14	0.68		6.2	9.9			0	0	7.2	7.8									
		2	14–27	0.65		4.3	8.7			0	0	5.9	7.3									
			45															0	0	98.7		
4	R. O.			Priming infusion = 4.0 ml. of 10% inulin, 4.0 ml. of 10% $Na_2S_2O_3$ and 2.5 ml. of 20% PAH																		
	3–8–53		76–219	Sustaining infusion = 0.5 Gm. inulin, 0.5 Gm. $Na_2S_2O_3$, 0.7 Gm. PAH and 14.6 mEq. NaCl per 100 ml., given at 0.5 ml. per min.																		
	Age: 22 days																					
	Wt.: 2097 Gm.		149															55.6	31.1	105.7	3.6	
	SA: 0.160 M^2	3	153–175	0.25	4.8	39.6	12.3			21.3	16.7	8.6	8.6									
		4	175–193	0.43	5.7	75.8	14.5			22.9	17.9	27.7	11.0									
		5	193–212	0.57	5.5	73.2	11.0			21.8	16.9	27.1	9.9									
			219															50.8	28.2	106.6	3.6	

TABLE I—*Continued*

Observation number	Subject	Period	Elapsed time	Urine flow	Inulin clearance	Urine										Blood						
						Na	K	NH_4	TA	PAH	S_2O_3	Cl	HPO_4	HCO_3	pH	Na	K	PAH	S_2O_3	Cl	HPO_4	HCO_3
			min.	*ml./min.*	*ml./min.*	*μEq./min.*										*mEq./L.*						
5	S. A. 4–28–53 Age: 33 days Wt.: 2159 Gm. SA: 0.164 M^2	1	0–16	0.14		4.9	5.7			0	0	6.3	5.0									
		2	16–32	0.34		8.7	10.3			0	0	10.4	10.1									
			35													136.6	6.3			110.8		
			78–175	Priming infusion = 4.4 ml. of 10% inulin, 5.5 ml. of 10% $Na_2S_2O_3$ and 2.8 ml. of 20% PAH Sustaining infusion = 0.5 Gm. inulin, 0.63 Gm. $Na_2S_2O_3$, 0.75 Gm. PAH and 14.4 mEq. NaCl per 100 ml., given at 0.5 ml. per min.																	
			141													142.0	5.8	57.5	43.3			
		3	144–162	0.31	5.2	51.5	19.0			19.3	23.8	12.4	11.2									
		4	162–177	0.43	5.4	78.2	15.8			17.2	23.2	30.7	11.8									
			178													145.4	5.3	48.3	40.5	115.7		
6	B. R. 5–12–53 Age: 12 days Wt.: 2171 Gm. SA: 0.165 M^2	1	0–16	0.24		4.1	6.0					7.2	5.6									
		2	16–30	0.45		5.5	5.9					8.0	4.9									
			34													137.8	5.6					
			43–229	Priming infusion = 4.4 ml. of 10% inulin, 5.5 ml. of 10% $Na_2S_2O_3$ and 2.8 ml. of 20% PAH Sustaining infusion = 0.5 Gm. inulin, 0.63 Gm. $Na_2S_2O_3$, 0.75 Gm. PAH and 14.4 mEq. NaCl per 100 ml., given at 0.5 ml. per min.																	
			105													143.0		64.0	46.1			
		3	109–139	0.21	4.8	48.0	7.8			20.4	23.7	10.7	9.7									
		4	139–169	0.23	4.9	51.7	8.7			19.2	22.8	11.0	10.5									
			171													142.4	4.7	54.8	40.7			
7	P. U. 5–19–53 Age: 34 days Wt.: 1964 Gm. SA: 0.152 M^2	1	0–44	0.17		2.3	8.3	0.9	1.9	0	0	5.0	3.6	0.5	6.1							
			49													141.1	5.4		0	103.3	3.9	25.9
			55–208	Priming infusion = 3.9 ml. of 10% inulin, 4.9 ml. of 10% $Na_2S_2O_3$ and 2.4 ml. of 20% PAH Sustaining infusion = 0.5 Gm. inulin, 0.63 Gm. $Na_2S_2O_3$, 0.75 Gm. PAH and 14.4 mEq. NaCl per 100 ml., given at 0.5 ml. per min.																	
			127													146.2		59.6	42.8	106.1		
		2	124–154	0.19	4.4	37.8	14.7	1.3	5.2	17.3	21.0	5.3	7.6	0.3	5.0							
		3	154–180	0.30	5.0	56.3	15.0	1.3	3.8	17.9	22.7	14.7	9.6	1.0	6.1							
		4	180–206	0.27	4.2	50.3	13.5	1.3	3.3	15.7	19.5	13.6	9.4	0.9	6.0							
			208													145.6	4.7	53.6	38.2	108.0	3.7	25.9
8	M. A. 12–2–52 Age: 13 days Wt.: 2212 Gm. SA: 0.167 M^2	1	0–20	0.49		10.8	15.1	1.3	2.5			14.8	7.3	1.5	6.5							
			22														6.2					29.5
			22–167	Sustaining infusion = 0.4 Gm. inulin and 16.0 mEq. NaCl per 100 ml., given at 0.5 ml. per min.																		
		2	82–139	0.18	3.6	7.0	6.4	2.3	3.7			9.4	5.6	0.3	5.2							
			114													142.6						26.3
		3	139–166	0.30	4.7	24.7	8.1	2.0	2.9			26.8	6.4	0.7	6.0							
			164																	105.6		27.1

urine. As indicated in the protocols, not all of these measurements were made in each observation.

GFR was estimated in each observation from the clearance of inulin (C_{IN}) during two or three urine collection periods following equilibration. Rates of excretion of electrolytes were calculated as microequivalents (μEq.) per min. Because of variation in the duration of the periods, mean values were calculated by weighting each value by the duration of the period.

RESULTS

The results of a typical observation are shown graphically in Figure 1 and protocols for each of the eight observations are given in Table I. Values for C_{IN} in this group of premature infants ranged from 3.6 to 6.5 with a mean of 4.8 ml. per min. Per 1.73 M^2, these values represent a range from 32 to 59 per cent of the adult mean value and are similar to those previously reported (9). The administration of sodium salts of PAH and S_2O_3 altered the renal excretion of electrolytes as follows:

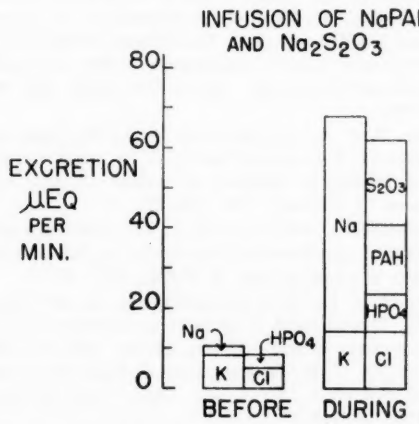

FIG. 1. EFFECT OF INFUSION OF UNREABSORBED ANIONS ON ELECTROLYTE EXCRETION IN A PREMATURE INFANT (SUBJECT P. U.)

Effect on sodium and potassium excretion

In each of the five observations (3–7) where sodium salts of PAH and S_2O_3 were given, there was an increase during the infusion in the absolute rates of excretion of sodium and potassium. However, the increase in rate of excretion of potassium was not even sufficient to cover the increased rate of excretion of chloride and phosphate which made up only 39 per cent of the total measured [3] anions excreted during the infusion. The major fraction (mean: 61 per cent) of the total measured anions excreted during the infusions consisted of PAH and S_2O_3 and the increase in the absolute rate of excretion of sodium was more than sufficient to cover these unreabsorbed anions. Thus, during the control periods the per cent of anions (calculated as the sum of Cl and HPO_4) covered by sodium ranged from 30 to 43 per cent and by potassium from 46 to 97 per cent. During infusions of PAH and S_2O_3, the per cent of anions (calculated as the sum of Cl, HPO_4, PAH, and S_2O_3) covered by sodium ranged from 68 to 87 per cent and by potassium from 13 to 25 per cent. The per cent of anions covered by sodium increased from a mean value of 35 per cent during the control periods to 80 per cent during the infusions while the per cent covered by potassium decreased from 63 to 21 per cent.

In the one observation (Subject 8) in which only sodium chloride was given, the changes in the per cent of anions covered by sodium and potassium were in the same direction as those which occurred during the infusion of PAH and S_2O_3. However, as expected, the magnitude of the changes was smaller.

Effect on other electrolytes

The rate of excretion of chloride increased in each observation. Although the rate of excretion of phosphate during PAH administration did increase over the control value in six of seven observations, in only one instance was the increase greater than two-fold.[4] Where measured, there were no marked and consistent changes in pH of the urine or in the rates of excretion of ammonia (NH_4), titratable acid (TA) or bicarbonate (HCO_3).

[3] During infusions of thiosulfate, sulfate (24) which is excreted at a high rate, was not measured.

[4] West and Rapoport (25) have reported 2.3 to 16-fold increases in the rate of excretion of phosphate following injections of PAH. However, their observations were made immediately after the injection of single large doses of PAH whereas here measurements were made during a continuous infusion and after a priming dose and equilibration period.

DISCUSSION

The mechanism by which, under certain conditions, the renal excretion of unreabsorbed anions (given as sodium salts) is covered principally by potassium rather than sodium is not clear. The present observations show that, in spite of a low GFR, the premature infant, apparently unlike the dog with an equivalent reduction in GFR induced acutely, excretes these anions covered almost wholly by sodium. Available data suggest that excretion of PAH and S_2O_3 covered principally by sodium is not due to any specific limitation in rates of potassium excretion in the premature infant. The highest rate of potassium excretion observed in these infants was 19.6 μEq. per min. representing 45 per cent of the rate of filtration of potassium. Under other conditions in a similar group of premature infants, Tudvad, McNamara, and Barnett (26) observed rates of potassium excretion as high as 34 and 36 μEq. per min., the former representing a value of 172 per cent of the rate of filtration.

SUMMARY

In the premature infant, as in the adult, the administration of sodium salts of the unreabsorbed anions, PAH and S_2O_3, results in the renal excretion of the anions covered principally by sodium.

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HEPATIC CIRRHOSIS: EFFECTS OF SODIUM CHLORIDE ADMINISTRATION AND RESTRICTION AND OF ABDOMINAL PARACENTESIS ON ELECTROLYTE AND WATER BALANCE [1, 2]

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(Submitted for publication July 14, 1953; accepted January 13, 1954)

The formation of ascites and edema by patients with chronic liver disease can be controlled by rigid dietary sodium restriction (1–5). When sodium is administered, ascites formation re-commences while sodium excretion usually remains minimal. These facts have not yet been integrated with the factors usually considered contributory to the formation of ascites and edema, *viz.:* elevated portal pressure (6, 7), reduced serum albumin concentration and colloid osmotic pressure (8–12), increased anti-diuretic activity (13), altered membrane permeability (4, 14), or altered renal tubular function (15). However, whatever the pathogenetic factors operating, fluid retention is readily conditioned by the sodium content of the diet.

The observations reported here concern patients with cirrhosis of the liver with ascites and edema in whom the alterations in electrolyte and water balance attributable to abdominal paracenteses and to sodium chloride administration and restriction were studied under controlled conditions of food, water, and sodium chloride intake.

MATERIALS AND METHODS

Seven patients with chronic advanced cirrhosis of the liver with edema and ascites requiring paracentesis were selected for study. The diagnosis in each case was established by history, physical examination, and laboratory tests of liver function. Six of these patients who were chronic alcoholics presented the classical findings of cirrhosis of the liver. No predisposing factor could be established in the seventh patient (H.O.). The clinical data of these patients are given in Table I.

The patients were studied on the Thorndike metabolic ward and were ambulatory. They were maintained on a constant basal diet which provided 75 grams of protein and at least 2800 calories daily.[4] This diet contained 10 mEq. of sodium and 104 mEq. of potassium by analysis.

Five of the patients (R. K., W. C., H. O., B. K., and B. B.) were initially observed for from two to four weeks during which 103 mEq. of sodium (6.0 Gm. sodium chloride) were added to the basal sodium restricted diet. In addition, W. C. was studied during a subsequent three-week period while receiving 51.5 mEq. of added sodium. After this initial period, the supplemental sodium was stopped, and the observations continued for two to three months on the sodium restricted diet (10 mEq. daily). Patients J. G. and C. M. were maintained on sodium restricted regimens throughout their studies.

The patients were weighed at the same time each day under constant conditions. Daily fluid intakes were recorded and maintained relatively constant throughout each study. Total daily urine outputs were measured and suitable aliquots taken for determination of sodium and potassium. Serial measurements of hematocrit and serum sodium were made; frequent determinations were done in the immediate pre- and post-paracentesis periods. The blood specimens were obtained without stasis from an antecubital vein four hours after breakfast and following a one-hour period of bed rest. Abdominal paracenteses were executed when indicated. The volume of fluid removed was measured, and its sodium content determined. Daily clinical estimates were made of the

[1] This investigation was sponsored by the Commission on Liver Disease, Armed Forces Epidemiological Board, and was supported in part by a grant from the Office of The Surgeon General, United States Army, Washington, D.C., and in part by grants from Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, and from Merck and Company, Rahway, New Jersey, to Harvard University.

[2] A preliminary report of this investigation was included in the Proceedings of the Forty-Second Annual Meeting of the American Society for Clinical Investigation, May 1, 1950 (J. Clin. Invest., 1950, **29**, 814).

[3] Welch Fellow in Internal Medicine of the National Research Council. This investigation was accomplished in part during the tenure of a United States Public Health Service Postdoctorate Fellowship.

[4] Lonalac, used in these diets as a salt-poor milk substitute, was generously provided by the Mead Johnson Company, Evansville, Indiana.

TABLE I

Clinical and laboratory findings in patients with cirrhosis of the liver

Patient, age and sex	Liver size*	Spleen size*	Abdominal collateral veins†	Esophageal varices x-ray‡	Serum albumin	BSP % retention	Duration of ascites	Number abdominal paracenteses	Average volume ascitic fluid removed	Average interval between paracenteses
					Gm. %		*months*		*liters*	*days*
R. K., ♂ 43	4	2	4+	P	2.1	36	6	9	10.9	21
W. C., ♂ 70	N.P.§	N.P.	3+	P	2.5	25	3	12	7.7	11
H. O., ♂ 62	14	4	2+	P	2.0	5	3	12	7.7	9
B. K., ♂ 53	14	7	2+	P	1.5	27	8	18	11.2	15
B. B., ♂ 47	7	4	4+	A	2.0	25	2	3	14.3	31
J. G., ♂ 46	4	N.P.	4+	P	2.0	42	0.5	1	15.0	—
C. M., ♂ 48	1	N.P.	1+	P	1.4	—	9	9	13.5	30

* cm. below costal margin.
† 0 to 4+.
‡ P = present, A = absent.
§ N.P. = not palpable.

amount of ascites and edema which were graded on a 0 to 4+ scale.

In the early studies, sodium determinations of urine, serum, and ascitic fluid were done by the method of Consolazio and Dill (16), and in the more recent studies sodium and potassium concentrations were analyzed by internal standard flame photometer (17, 18). Fecal and sweat losses of sodium were assumed to be 10 mEq. daily (19–23).

Bromsulphalein retention was measured 45 minutes after the intravenous injection of 5 mg. of the dye per kilogram of body weight (24). Serum albumin concentration was determined by an immunological method which utilized antihuman albumin horse serum (25).

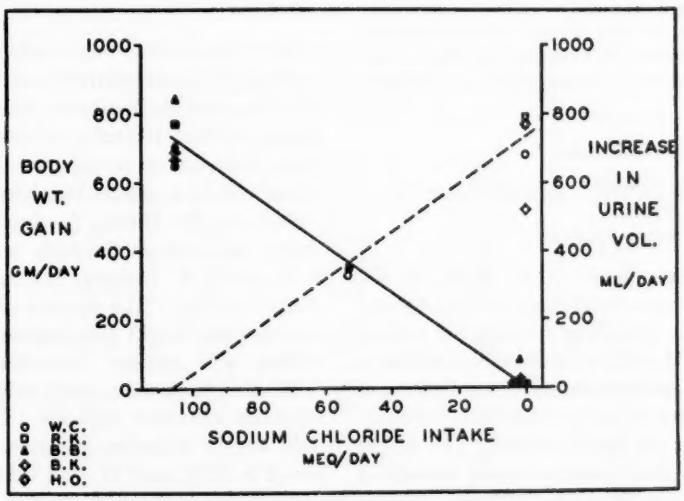

FIGURE 1

The solid diagonal line indicates the "theoretical" body weight gain expected with the isotonic retention of sodium added to the basal diet. The dashed line indicates the "theoretical" increase in urine volume expected upon withdrawing sodium from the diet.

TABLE II

Effect of sodium administration and restriction on body weight and urine volume

Patient	Days of study	Sodium intake mEq./day		Fluid intake average	Urine output average	Increase in urine volume during sodium restriction average	Body weight gain average*	Urine sodium average
		Diet	Added					
				ml./day	ml./day	ml./day	Gm./day	mEq./day
R. K.	1–28	10	103	2220	1337		783	1.0
	29–43	10	0	2290	2130	793	0	0.7
W. C.	1–14	10	103	2220	808		649	2.9
	15–34	10	51.5	2210	1137	329	341	1.6
	35–49	10	0	2225	1487	679	8	3.1
H. O.	1–13	10	103	2620	1606		673	1.0
	14–23	10	0	2720	2380	774	0	1.0
B. K.	1–14	10	103	1380	668		666	0.6
	15–35	10	0	1360	1180	512	0	1.6
B. B.	1–13	10	103	2380	979		691	0.8
	14–29	10	103	2030	967		845	0.6
	30–44	10	0	1890	1197	†	93‡	0.7

* The expected gains in body weight with the isotonic retention of the quantities of sodium added to the basal diet are 736 Gm. and 368 Gm., respectively.

† Calculation not made because of marked fluctuations in fluid intake.

‡ A total body weight gain of 295 grams during this period of study occurred during the five days immediately following paracentesis on day 30.

Blood hematocrit was determined by the method of Wintrobe (26).

These studies were done during the winter and spring months so that variations in skin losses of water and sodium were minimal.

The patients studied presented no clinical or laboratory evidence of cardiac or renal disease, nor was any patient's course complicated by vomiting, diarrhea, or gastrointestinal bleeding, except in patient B. B. as indicated below.

RESULTS

I. Sodium Chloride Administration

A. Sodium and water balances

The five patients (R. K., B. B., B. K., H. O., and W. C.) who were maintained for two to four weeks on the diet providing 10 mEq. of sodium daily to which 103 mEq. of sodium was added as sodium chloride, accumulated ascites and edema at a rate (as measured by body weight gain) averaging 720 grams per day for all studies. The weight gains for each of these patients during periods of sodium ingestion are shown in Table II. This is similar to the weight gain expected if the 103 mEq. of added sodium were retained isotonically (140 mEq. per L.), namely 736 grams per day (Figure 1). A similar relationship was observed in W. C. (Table II) while he ingested 51.5 mEq. of sodium daily in addition to the basal diet.

B. Physiologic alterations attributable to paracentesis

These observations were made while the patients continued to ingest relatively constant fluid intakes, and diets constant in calories, nitrogen and sodium content to which 103 mEq. of sodium chloride were added daily, except in the case of C. M. who was maintained on a sodium restricted regimen.

Body weight: During the first several days following paracentesis the body weights of R. K., B. B., and B. K. (Figures 2, 3, and Table III) increased rapidly. The amount of weight increase exceeded the weight gain expected if the ingested sodium was retained isotonically with water. C. M. also displayed a rapid weight gain while on a sodium restricted regimen. This alteration in body weight following paracentesis was not observed in H. O. and W. C. (Table III).

Ascites and edema: Following paracentesis, ascites rapidly reaccumulated, and edema decreased. Some patients who had moderate to marked pitting edema of the lower extremities were free of edema or displayed marked decreases in the amount of

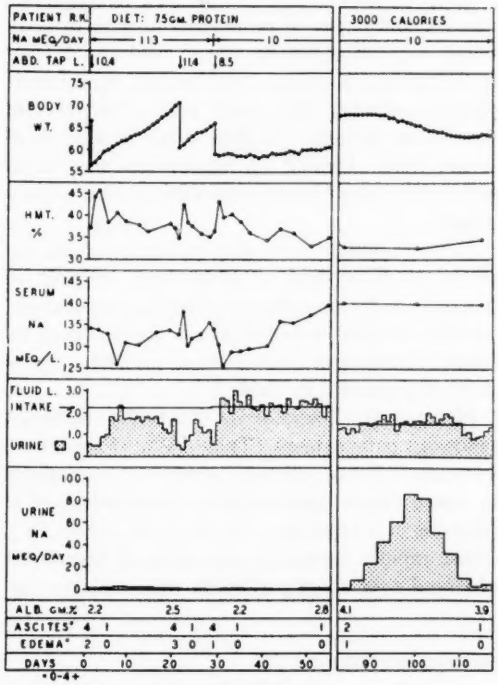

FIGURE 2

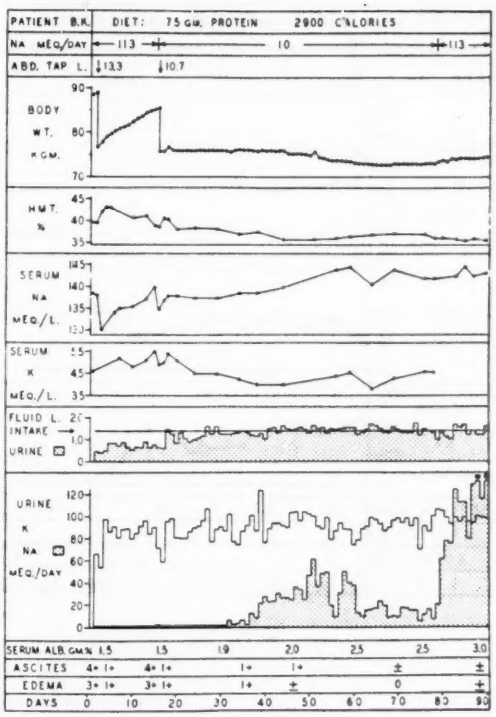

FIGURE 3

TABLE III

Effect of paracentesis on body weight and urine volume

Patient	Days of study	Sodium intake	Fluid intake average	Urine output average	Body weight gain average	Urine sodium average
		mEq./day	*ml./day*	*ml./day*	*Gm./day*	*mEq./day*
R. K.	1*–3	113	1740	527	1061	0.9
	4–20	113	2330	1623	682	1.2
	21*–23	113	2100	520	1023	0.3
	24–28	113	2130	1140	727	0.8
W. C.	1*–3	113	2160	863	750	9.9
	4–14	113	2240	792	610	1.0
H. O.	1*–3	113	2550	1540	633	0.8
	4–13	113	2640	1636	690	1.0
B. K.	1*–3	113	1380	467	1080	0.5
	4–14	113	1380	723	578	0.6
B. B.	14*–16	113	2000	745	1098	0.5
	17–29	113	2030	1000	787	0.6
C. M.	1*–3	10	3600	875	900	—
	4–9	10	3700	2165	0	—

* Day on which abdominal paracentesis was done.

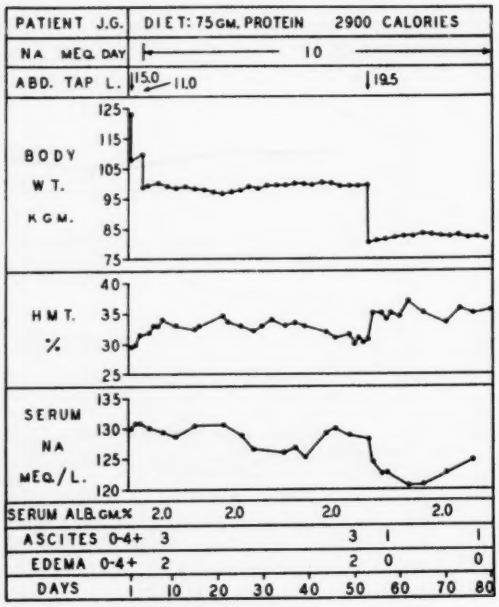

FIGURE 4

edema within several days after abdominal paracentesis (Figures 2, 3, 4). During this time ascites expanded out of proportion to gain in body weight suggesting that the edema fluid was shifting to the peritoneal cavity. Abdominal paracentesis became necessary again after only a few days in some of the patients. In these cases the amount of ascitic fluid obtained on repeat tap was much greater than could be accounted for by gain in body weight.

Urine volumes: Reduction in urine volume occurred on those days on which body weights increased rapidly immediately following paracentesis. Urinary sodium excretion which was minimal remained unchanged during these periods. These data are presented in Table III.

Hematocrit: Hematocrits frequently increased following paracenteses (Table IV). Progressive increases in hematocrit were noted in those patients in whom repeated paracenteses were necessary at intervals of several days (R. K., J. G., Table IV). These rapidly occurring alterations in hematocrit observed over short periods of time indicate (as-

TABLE IV

Alterations in hematocrit following paracentesis

Patient	Date of tap	Hct. on day of tap	Days following paracentesis							
			1	2	3	4	5	6	7	8
R. K.	1/22/49	37	40	41	44	42	43*			
	1/27/49	43	45	46		43			40	
	3/25/49				43	39	37*			
	3/31/49†	37	44	46		39		41		39
	4/20/49	34	43	38	38		36		35	36*
	4/28/49‡	37	43	40		40		39		36
W. C.	1/22/49	40	43	41					40	
	2/5/49	36	42		39		37		38	
	2/25/49‡	37	38	40	39	37		38		
	3/13/49§	37	39	38	41	42	42		40	
H. O.	12/1/49	42	43	45		46	45	44		
	12/15/49‡	42	43	42		40	42	38		
B. K.	1/5/50	40	42	43		43	40	40		41
	1/19/50‡	39	41	40		38		40		38
B. B.	12/21/49	27	29	29			27		29	
	1/6/50	26	27	27	28	28		28	27	
J. G.	12/20/49‡	30	30	32	32*					
	12/23/49	32	33	33	34	31		31	33	
	2/10/50	31	36	34	35	34	35		35	
C. M.	6/7/48‡	40	47	44					42	
	11/5/48	34	37		38					

* A paracentesis necessary on this day because of a shift in edema fluid.
† Hematocrit data following this date are included in Figure 2.
‡ Sodium restricted diet instituted.
§ Spontaneous rupture of umbilical hernia with drainage of ascitic fluid for three days.

TABLE V

Sodium balance data during periods of sodium administration and sodium restriction

Patient	Days of study	Day of tap	Volume ascitic fluid	Na ascitic fluid	Na serum	Total ascitic Na	Days of study	Sodium, *mEq./day*							
								Output				Intake			
								Ascitic	Extra-renal	Urine	Total	Diet	Added	Total	Balance
			ml.	*mEq./L.*	*mEq./L.*	*mEq.*									
R. K.	1–28	1	10,350	134	134										
		21	11,375	137	138										
		29	8,500	132	132	2,680	28	96	10	1	107	10	103	113	+ 6
	29–43						15	0	10	1	11	10	0	10	− 1
W. C.	1–14	1	8,900	146	143										
		15	8,575	137	138	1,175	14	84	10	3	97	10	103	113	+16
	15–34	35	8,600	139	141	1,195	20	60	10	2	72	10	52	62	−10
	35–49						15	0	10	3	13	10	0	10	− 3
H. O.	1–13	1	9,700	133	132										
		14	8,350	137	138	1,144	13	88	10	1	99	10	103	113	+14
	14–23						12	0	10	1	11	10	0	10	− 1
B. K.	1–14	1	13,300	139	138										
		13	10,680	136	135	1,452	14	104	10	1	115	10	103	113	− 2
	15–35						20	0	10	2	12	10	0	10	− 2
B. B.	14–29	14	15,000	134	129										
		30	12,000	127	129	1,524	16	95	10	1	106	10	103	113	+ 7
	30–44						15	0	10	1	11	10	0	10	− 1

suming constant red cell mass) reductions of plasma volumes occurring after paracentesis. These reductions of plasma volume occurred in some cases while edema fluid was rapidly decreasing in dependent areas. In no instance did the hematocrit fall below the control value in this post-paracentesis period. Hematocrits often returned to pre-paracentesis levels in four to eight days following paracentesis, but occasionally remained elevated for longer periods.

II. Sodium Chloride Restriction

A. Sodium and water balance

After the periods of sodium ingestion, the added sodium chloride was withdrawn and the basal diet continued. During the initial two to three week period of sodium restriction, accumulation of ascites was abruptly halted and no patient gained appreciable weight except during the first several days after paracentesis as described above. Urine volumes increased by amounts approximating the daily weight gains observed during periods of sodium ingestion and ranged from 467 ml. per day to 793 ml. per day, with an average of 680 ml. for all patients. This close relation between the rate of weight gain during sodium ingestion and the increases in urine volume resulting from withdrawal of sodium from the diet is indicated in Figure 1 and Table II.

These patients excreted only 0.6 to 3.1 mEq. of sodium in the urine daily, regardless of sodium intake. Losses of sodium in the feces in the absence of diarrhea are small and are affected little by marked changes in sodium intake (19). Fecal and sweat losses of sodium were assumed to be 10 mEq. daily (19–23). It is possible that sweat losses of sodium were even less than estimated since patients with ascites and subjects on a sodium restricted regimen have reduced sweat sodium concentrations (20, 23, 27). In contrast to the relatively small amount of the daily sodium intake excreted in the urine, stool and sweat, large losses of body sodium occurred at times of abdominal paracenteses, since the sodium content of the ascitic fluid removed approximated the so-

dium concentration of the serum. These large losses of sodium entailed by a paracentesis amounted to from 1142 mEq. to 2678 mEq. Calculations of approximate sodium balances, taking into consideration the losses entailed by paracentesis, indicate that the patients studied are in approximate sodium equilibrium (Table V).

B. Serum sodium

Serum sodium concentrations in 11 patients with cirrhosis of the liver without ascites and edema ranged from 137 to 146 mEq. per L. and averaged 140 mEq. per L., values comparable to those found in 18 normal subjects (range 137 to 146, average 141 mEq. per L). Thirty-four patients with ascites in whom this measurement was made shortly after hospitalization and prior to the institution of diuretic therapy, dietary sodium restriction, or paracentesis frequently demonstrated hyponatremia (range 128 to 144, average 137 mEq. per L.). The lowest average serum sodium value was observed in 17 patients who had had repeated recent paracenteses (range 121 to 143, average 132 mEq. per L.). Although occasionally normal values were obtained, the serum sodium concentrations measured in the patients studied serially were frequently below normal, and the hyponatremia which was often present was more marked in some patients immediately following abdominal paracentesis (Figures 2, 3, 4). The degree of post-paracentesis hyponatremia and the time at which it occurred may be related to the amount of peripheral edema available for mobilization and to the rate of expansion of the ascites. Some patients with massive edema were less likely to display a decrease in serum sodium concentration following paracentesis than those with little or no edema. The relation of hyponatremia to the other physiologic alterations occurring immediately following paracentesis is discussed below.

C. Prolonged sodium restriction

Six patients were further studied in the hospital for periods of 60 to 90 days during which the diet rigidly restricted in sodium content was continued. The need for paracentesis was alleviated by continuing the diet providing 10 mEq. of sodium daily. Body weights remained the same or increased slowly but ascites and edema did not reaccumulate. Concurrently, there was gradual improvement in their clinical nutritional status, and in most instances the concentration of albumin in the serum increased. In three patients (R. K., Figure 2; B. K., Figure 3; and H. O.) serial hematocrits progressively decreased to values well below those observed early in the studies or immediately following paracentesis. These alterations in hematocrit were paralleled by changes in hemoglobin concentration, so that they could not be attributed to changes in the size of the red blood cells; nor could they be accounted for by blood loss (guaiac tests on all stools were negative). Serial measurements of serum sodium concentrations in these three patients tended to rise with the continued control of ascites formation by dietary sodium restriction. As the hematocrit gradually decreased and the serum albumin and serum sodium values approached the normal range, there occurred a significant increase in the urinary excretion of sodium. This was most marked in patients R. K. (Figure 2) and B. K. (Figure 3) who underwent diureses with losses of weight and excretion of up to 70 mEq. and 85 mEq., respectively, daily, although only 10 mEq. was being ingested. The time at which significant quantities of sodium appeared in the urine following the institution of the low sodium diet varied from about two to eight weeks.

During a final period of study in patient B. K. (Figure 3) 103 mEq. of sodium were again given in addition to the basal diet. In contrast to the initial period of study, gain in body weight did not occur and a quantity of sodium approximating that ingested was excreted in the urine. The marked increase in the excretion of sodium in the urine which occurred after the ingestion of sodium was not associated with alteration in the level of urinary potassium excretion.

Two of the patients (J. G., Figure 4 and B. B.) failed to excrete sodium in the urine during 80 and 40 days of observation, respectively. In these instances the serum sodium concentrations were consistently low, and in the case of J. G. the hematocrit increased following paracentesis early in this study and remained above the initially observed values. The hematocrit in B. B. generally tended to decrease, but this is not possible to interpret since her course was complicated by intermittent

episodes of mild to moderate gastro-intestinal bleeding.

No untoward effects were observed in the patients during the prolonged periods of dietary sodium restriction. The incidence of cramps in the legs and fingers was less during the salt restriction periods than when sodium was provided and frequent paracenteses were necessary.

DISCUSSION

Immediately after paracentesis the following phenomena were observed: a rising hematocrit, rapidly expanding ascites, a decrease in peripheral edema, reduction in urine volume, no change in the minimal urinary sodium excretion, and hyponatremia. These observations can be explained by a rapid transudation of fluid from the circulating plasma into the peritoneal cavity following the reduction of intra-abdominal pressure after paracentesis. Mobilization of edema fluid is attributable to reduction in femoral venous pressure which occurs following paracentesis (7). The post-paracentesis hemoconcentration is accompanied by oliguria and water retention (antidiuresis). Since sodium balance remains unchanged following paracentesis (constant intake and unchanged minimal excretion), the water retention in the immediate post-paracentesis period results in dilution of extracellular fluid and hyponatremia. The retention of water without physiologically equivalent quantities of sodium at this time is corroborated by gains in body weight which were in excess of those expected if sodium was being retained isotonically. That this retention of water may be great enough to produce a symptomatic hyponatremia following paracentesis has been reported (28).

The prevention of the accumulation of ascites and edema by dietary sodium restriction was accompanied in several patients by gradual, but progressive, reductions in hematocrit and increases in serum sodium concentration. When the serum sodium concentration increased to or near to normal, excretion of sodium in the urine recommenced. The source of the sodium for the observed rise in serum sodium concentration, which has also been reported by others (27), in the face of progressive hemodilution, is not clear especially since these patients were on a diet virtually free of sodium. An intracellular exchange of sodium for potassium may be postulated such as usually is expected following rehydration (29, 30, 31). However, potassium balance failed to become more positive during this period in the one patient so studied (B. K., Figure 3). Thus, the data, at present limited, do not substantiate this interpretation. Secondly, it is possible that the sodium diuresis may be preceded by a water diuresis. That this may occur is supported by observation of two patients with cirrhosis and ascites who had an initial hyponatremia and who spontaneously underwent a diuresis. These patients displayed increases in serum sodium concentration and losses in body weight prior to the appearance of increases in urinary sodium excretion, suggesting that a diuresis of water preceded that of sodium (32).

The changes observed during prolonged dietary sodium restriction may be related to improvement in liver function and in general nutritional status attributable to the prolonged administration of a nutritious diet (33), the control of ascites formation and the relative degrees of inactivity necessarily accompanying periods of hospitalization.

The results of the present study indicate that abdominal paracentesis leads to a reduction in plasma volume and that repeated paracenteses may result in a chronic state of hemoconcentration and of hyponatremia. Although this interpretation of the data does not explain the genesis of ascites and edema in patients with severe liver disease, it is possible that the initial formation of ascites and edema may lead to a decrease in plasma volume similar in mechanism to that which develops acutely following paracentesis. This would imply that elevated portal pressure and hypoalbuminemia first lead to transudation of fluid from the circulating blood into the peritoneal cavity. The resulting decrease in plasma volume might then provide the stimulus for the appearance of increased antidiuretic activity (34, 35), and for the renal conservation of sodium (36, 37). The findings of increased antidiuretic activity (13) and decreased quantities of sodium (2–5) in the urine would then be physiologic homeostatic responses. That this may be the case is supported by evidence that patients with liver disease metabolize normally endogenous or physiologic doses of exogenous posterior pituitary hormone (38–40), and by the observations in the present study that progressive

decreases in hematocrit preceded the onset of a diuresis. The validity of the hypothesis that the effective plasma volume [5] is reduced in patients with cirrhosis of the liver and ascites awaits the development of methods for making this measurement accurately, particularly in patients with alterations in their plasma proteins, and in their portal vascular beds.

SUMMARY AND CONCLUSIONS

The effects of sodium chloride administration and restriction and of abdominal paracentesis on electrolyte and water balance were studied in seven patients with cirrhosis of the liver and ascites.

During periods of sodium chloride ingestion, ascites and edema accumulated at a rate approximating that expected if the salt was retained isotonically. When a diet restricted in sodium was provided subsequently the accumulation of fluid was abruptly halted, and daily urine volumes increased by amounts approximating the daily weight gains previously observed during periods of sodium ingestion. With continued dietary sodium restriction improvement in clinical nutritional status, a progressive rise in the serum albumin and sodium concentrations and fall in hematocrit usually occurred. Gradual diuresis with loss of ascites and edema was found to accompany these changes.

Immediately following abdominal paracentesis sodium was not retained isotonically in some patients. Water was retained in excess of salt. This phenomenon is explained as follows: After paracentesis hemoconcentration occurs. The patient responds to this by antidiuresis. Since sodium is already being maximally retained, the resulting water retention contributes to hyponatremia which is most marked following paracentesis.

The studies presented suggest that the effective systemic plasma volume is decreased in patients with cirrhosis of the liver and ascites who have had repeated paracenteses; and that the increased antidiuretic activity and the decreased sodium content of the urine of these patients may be, in part at least, the result of homeostatic responses initiated by abdominal paracentesis, and also possibly by the initial formation of ascites.

[5] Systemic plasma volume, exclusive of that contained in the portal vascular bed.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of the Misses Alice Ballou, Virginia Olsson, and Ellen Doyle, Mrs. Frederick S. Bigelow and Mrs. Frederick Hannon, and the dietetic assistance of Mrs. J. Spencer Rochefort and Miss Barbara Wolfe.

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GENETIC LINKAGE BETWEEN OVALOCYTOSIS AND THE RH BLOOD TYPE [1]

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(Submitted for publication December 7, 1953; accepted January 20, 1954)

The presence of elliptical or oval erythrocytes in peripheral blood has been noted in various racial groups since its first accurate description by Dresbach (1) in 1904. Hunter and Adams (2) showed that ovalocytosis was inherited, and Cheney (3) established the pattern of inheritance as that of a simple autosomal Mendelian dominant. Early interest in this anomaly centered around the possibility that the trait might serve as an explanation for a hemolytic anemia. Recently, interest in genetic linkage relationships of such hereditary erythrocytic traits has arisen. Information as to genetic linkage may provide a method of predicting the occurrence of hereditary diseases.

In the summer of 1952, when a patient with ovalocytosis came to our attention, a study of her entire family was undertaken in an attempt to demonstrate a clear-cut case of linkage in this dominant trait. We proposed two questions: *1.* Is there any linkage between the genes for ovalocytosis and those which determine the antigens in the various blood groups? *2.* Do the numbers of oval erythrocytes have any bearing on the occurrence of a hemolytic anemia?

CASE MATERIALS AND METHODS

Figure 1 is the pedigree of the Family R. Twenty-eight members of this family and three spouses were studied. Dried blood films were prepared from venous or shed blood to demonstrate the presence or absence of ovalocytes and their approximate number in those affected. Slides were read independently by at least two of the authors. Reticulocytes were counted in dry blood films. Hematocrit and serum bilirubin were determined according to techniques used by Wintrobe (4) with heparin as the anti-coagulant. Affected members were compared with their normal siblings.

The marker genes chosen for the linkage study are those which determine the blood group antigens for the ABO, MNs, Rh, Lewis, Kell, and Duffy systems. The genetic basis of these groups is well established and they can be accurately and readily determined. Saline cell suspensions were prepared from clotted blood in determining these blood groups using the test tube method. Complete serial typing was done on the 22 family members and the three spouses.

RESULTS

The data pertaining to ovalocytosis and to the blood types are tabulated in Appendix A. In twelve of 26 members of this family, 15 to 85 per cent of the erythrocytes were oval. Of the total family, only 18 individuals have affected parents, and only one, the proposita (III-6), showed any significant degree of reticulocytosis. The osmotic fragility and fecal urobilinogen on this patient were normal. When the indirect reacting serum bilirubin, the reticulocyte counts, and hematocrit determinations on persons affected with ovalocytosis were compared with their unaffected controls, there were no differences which are statistically significant in the small number of instances. These data are presented in Appendix B. Here, the percentage of oval cells expresses the average of multiple determinations. In the other column, where more than one test was run, the lowest hematocrit is recorded, the highest reticulocyte count and the most abnormal van den Berg. There was no past history of hemolytic anemia in any of these individuals.

The results of the blood typing data on the ABO, MNs, Lewis, Kell, and Duffy systems agree with other investigators (5–7) in that there is no indication of close linkage between the genes of these systems and the gene for ovalocytosis. Inspection of Figure 1 reveals that in each of the eleven individuals on whom complete data were obtained, the chromosomal distribution for the oval traits

[1] Preliminary report was presented in part in Federation Proceedings, 12: 16, March, 1953; final report given before Midwestern Section, American Federation for Clinical Research, October 29, 1953.

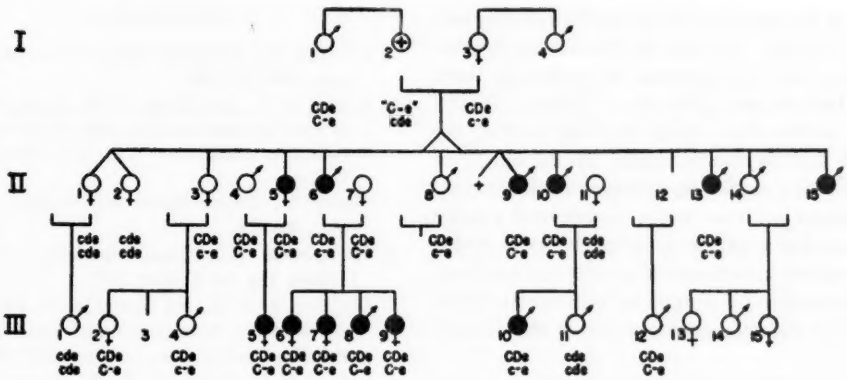

FIG. 1

and the Rh pattern CDe (R_1) are identical. This indicates genetic linkage. Of the unaffected members with affected parents, only two, II-3 and II-8, have this Rh pattern.

DISCUSSION

From these limited data we conclude that an increased proportion of elliptical erythrocytes in the peripheral blood is not accompanied by an increased tendency toward hemolysis in this family during the period studied. These data do not exclude the possibility of an episodic type of hemolytic anemia, nor do the number of examinations performed warrant a categorical opinion. It is possible that in ovalocytosis the expression of a clinically discernible anemia is dependent upon homozygosity as was found by Valentine and Neel (8, 9) for thalassemia major and minor. In order to test this hypothesis in ovalocytosis, it would be necessary to find homozygous persons. In view of the limited incidence of ovalocytosis as indicated by the studies of Wyandt, Bancroft, and Winship (10), this question will be difficult to resolve.

In examining the pedigree of Family R (Figure 1), two members, *i.e.*, II-3 and II-8, who do not show the oval trait, have the chromosome represented by CDe(R_1) which was common to all individuals with ovalocytosis. These two members have affected parents, thus separating them from the remaining three normal persons showing the CDe representation. Such an occurrence can be explained by assuming that II-3 and II-8 inherited the CDe complex from the parent who did not exhibit ovalocytosis. Another possibility is that these two people represent cross-overs. Our study on linkage confirms the report of Goodall, Hendry, Lawler, and Stephen (6) of an independent, simultaneous investigation. Since the data of these English workers are more conclusive than those presented in this paper, we feel justified in concluding that in some families the gene for ovalocytosis is definitely on the same chromosome as are the genes for the Rh blood groups, and that the linkage is unusually close. In two other families reported by Chalmers and Lawler (5) the establishment of linkage between ovalocytes and the Rh blood group is equivocal. Such observations illustrate the lack of homogeneity in human linkage studies.

Although ovalocytosis in most cases is not a disease state, the establishment of a clear-cut case of linkage in an hereditary, human trait provides a new tool for clinical investigation. This will perhaps provide the method necessary to evaluate the role of genetic predisposition to such familial

diseases as certain types of idiopathic epilepsy and diabetes mellitus. To date the relative importance of heredity and environment in producing these diseases has not been properly evaluated. Identical twin studies have been the best method yet proposed, but these are limited by the rarity of identical twins raised in different environments. Close linkage with an easily determined genetic trait such as blood groups would allow one to evaluate the relative importance of genetic and environmental influences by comparing the results of the genetic type with the manifestation of the disease.

SUMMARY AND CONCLUSIONS

1. A large family in which ovalocytosis occurred as a dominant trait has been catalogued as to the blood type of the individual members. Evidence for a hemolytic anemia in affected members was sought, but not found.

2. On the basis of the data reported, it is postulated that the gene for ovalocytosis is on the same chromosome as are the genes for the Rh blood groups.

ACKNOWLEDGMENTS

We wish to thank Drs. Paul R. David and Lawrence H. Snyder for their advice and criticism, and Mr. Glen H. Hill of Ortho Research Foundation who kindly supplied some of the typing sera used.

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APPENDIX A—FAMILY "R"

Generation and number		Age, years	Average % ovalocytes	ABO	Rhesus	MNS	Lewis Le^a	Kell	Duffy Fy^a
I	1	70	0	0	CCD.ee	NS	−	−	..
	3	68	0	A1	CcD.ee	Ns	−	−	−
	4	70	0	..	..	..	..	..	..
II	1	47	0	0	ccddee	NS(?)	−	−	−
	2	47	0	0	ccddee	Ns	−	−	−
	3	45	0	0	CcD.ee	Ns	−	−	−
	4	40(?)	0	0	CCD.ee	MNS	+	..	−
	5	38	21	0	CCD.ee	NS	−	−	..
	6	39	33	A1	CcD.ee	NS	−	−	−
	7	38	0	A1	CCD.ee	Ns	−	−	−
	8	33	0	0	CcD.ee	Ns	−	−	−
	9	32	15	A1	CCD.ee	NS	−	−	..
	10	30	85	0	CcD.ee	NS	−	−	−
	11	28	0	B	ccddee	NS	−	..	−
	13	28	25	A1	CcD.ee	NS	−	..	−
	14	30	0	..	..	..	..	..	..
	15	19	35	..	..	..	..	..	..
III	1	23	0	0	ccddee	MNS	−	−	−
	2	24	0	B	CCD.ee	NS	−	−	−
	4	20	0	B	CcD.ee	MNS	−	..	−
	5	15	41	0	CCD.ee	MNS	−	−	..
	*6	16	30	A1	CCD.ee	NS	−	−	−
	7	13	15	A1	CCD.ee	NS	−	−	−
	8	9	18	A1	CCD.ee	NS	−	−	−
	9	6	48	A1	CCD.ee	NS(?)	−	−	−
	10	12	47	0	CcD.ee	NS	−	−	−
	11	9	0	0	ccddee	NS	−	−	−
	12	9	0	0	CCD.ee	MNs	−	−	−
	13	4	0	..	..	..	..	..	..
	14	3	0	A2	..	MN.	..	..	..
	15	2	0	A2	..	MN.	..	..	..

* Proposita.

APPENDIX B—FAMILY "R"

Generation and number		Average % ovalocytes	Hematocrit	Retics.	van den Berg		
					Total	Direct	Indirect
			%	%		*mg. %*	
II	10	85	..	0.5	..	..	..
III	9	48	32	1.3	..	..	..
III	10	47	..	0.8	..	..	..
III	5	41	..	0.4	..	..	..
II	15	35	..	0.9	0.61	0.30	0.31
II	6	33	47	1.3	0.85	0.56	0.29
*III	6	30	39	3.5	0.62	0.21	0.41
II	5	21	..	0.5	..	..	..
III	8	18	34	0.6	..	..	..
II	9	15	..	0.4	..	..	..
III	7	15	36	0.9	0.61	0.38	0.22
I	3	0	40	0.8	..	..	..
I	4	0	..	0.4	..	..	..
II	1	0	..	0.6	..	..	..
II	2	0	37	0.6	..	..	..
II	3	0	38	0.4	0.60	0.18	0.42
II	8	0	45	0.3	0.73	0.36	0.37
II	14	0	..	0.5	0.44	0.14	0.30
III	1	0	49	0.4	..	..	..
III	2	0	36	0.4	0.51	0.20	0.31
III	9	0	34	0.4	0.56	0.21	0.35
III	11	0	..	0.1	..	..	..
III	13	0	..	0.5	..	..	..
III	14	0	..	0.8	..	..	..
III	15	0	..	0.5	..	..	..

* Proposita.

BIOCHEMICAL STUDY OF MUSCLE IN PROGRESSIVE MUSCULAR DYSTROPHY [1]

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(Submitted for publication May 11, 1953; accepted January 13, 1954)

Current concepts of progressive muscular dystrophy hold that it is a disease in which, through genetic error, there are intrinsic defects in muscle metabolism, although the nature of these hypothetical defects remains unrecognized. It is the purpose of this report to describe one approach to the problem and to show that there are defects in the metabolism of isolated muscle from patients with progressive muscular dystrophy.

METHODS

Assessment of enzymatic activity of skeletal muscle obtained by biopsy presents several difficulties. In the first place, as in any quantitative chemical analysis of mixed tissues, there is the question of an adequate reference base, as discussed by Hoagland (1) and by other investigators (2). A specimen of muscle obtained by biopsy contains varying proportions of fatty tissue, non-muscular fibrous tissue, vascular tissue, extracellular water, and muscle fibers. Diseased muscle is particularly variable in its heterogeneity, and it has been important to the present study to estimate the degree of fibrous replacement of muscle in biopsy specimens. The reference base selected was non-collagen nitrogen (2), a term applied to nitrogen content of material soluble in dilute alkali, as distinguished from alkali-insoluble collagen and elastin.

A second difficulty associated with assessment of enzymatic activity results from inter-action of enzyme systems present in the muscle sample. Since purification of enzymes is not yet quantitative, it has been necessary to employ impure preparations as test systems. In multi-enzyme systems, such as the glycolytic series, the over-all rate is limited by a single slowest step. This renders quantitative assessment of any other step uncertain, since the rate of the reaction will be proportional to the quantity of enzyme only if that reaction is rate-limiting. The difficulty has been circumvented in the case of phosphorylase by Cori and Cori (3) by inhibiting the next enzyme in the chain, and in the case of aldolase by Sibley and Lehninger (4) by removing from the system the product of the reaction under study. Because suitable methods for their determination were available, these two enzymes, phosphorylase and aldolase were selected for study.

[1] Supported by grants from the Caisse Nationale de Sécurité Sociale (France), the Institut National d'Hygiène (France), and the Muscular Dystrophy Associations (U. S. A.).

In addition, establishment of non-collagen nitrogen as a reference base provided an opportunity to repeat previous observations on the non-heminic iron content of muscle. Earlier work from this laboratory (5, 6) showed that non-heminic iron (that is, iron not included in chromoproteins, myoglobin, and the cytochromes) occurred in muscle in two varieties; one extractable by reducing agents, the second apparently linked with myosin (7). It had been found that the extractable iron in muscle from dystrophic patients was low in terms either of wet weight of muscle or of fat-free dry weight (8–10).

Biopsies of muscle were obtained under general anesthesia. The samples were frozen at once in liquid nitrogen and analyses were performed without delay. Biopsies from patients without muscular dystrophy, controls, were obtained from the right transversus abdominis during operation for appendicitis. Biopsies from patients with muscular dystrophy were obtained from the left transversus abdominis. After freezing the muscle, an aqueous extract was prepared in the Potter-Elvehjem homogenizer held at the temperature of melting ice. The extracting medium was 20 volumes of 0.1 N sodium fluoride. Aliquots were analyzed for non-collagen nitrogen (11), for total nitrogen, for aldolase concurrently by the method of Dounce, Beyer, and Barnett (12, 13) and by the method of Sibley and Lehninger (4), and for phosphorylase by the Coris' method (3). In addition, in a number of patients the activity of aldolase in serum was also assessed (14). In three patients interpretation of values for non-collagen nitrogen were checked by determination of collagen and elastin (scleroproteins) by the method of Neumann and Logan (15). Extractable iron was determined colorimetrically with orthophenanthroline, using sodium dithionine as a reducing agent (16).

For phosphorylase assay the homogenate was centrifuged, the supernatant extract diluted with two volumes of a mixture of cysteine and NaF, pH 6.0, and left for two minutes at 38°. One-tenth ml. was used for the assay. Final concentration of cysteine was 0.05 M, pH 6.0. For aldolase assay the 1/20 extract was diluted with four volumes of water. Two-tenths ml. were used in the presence of 0.5 ml. 0.01 M fructose diphosphate, hydrazine and collidine buffer pH 7.5.

RESULTS

In Table I, collagen nitrogen is expressed as per cent of total nitrogen in the biopsy specimen. There was a definite increase in collagen nitrogen

TABLE I

Ratio of collagenous nitrogen to total nitrogen (in per cent)

Controls	Dystrophics
9.6	36
18.5	50
15	22
10	40
16.6	16
11	52
17	26
10	45
6	22
	33
	36
	50
	40
	21
	38
	35
	20
	33
Mean: 12.6 per cent±1.4	34.2 per cent±2.6

in muscle from patients with muscular dystrophy; the single patient in the high normal range was only slightly affected clinically. In general, the relative collagen nitrogen content was higher in those in whom the disease seemed most advanced, although it never exceeded half the total nitrogen even when enzymatic activity had virtually disappeared, as will be presented later.

Determination of collagen and elastin by the method of Neumann and Logan (15) agreed closely with the value obtained by the simpler method. The content of hydroxyproline in collagen, the amino acid upon which the measurement depends, was presumably the same in normal and dystrophic subjects.

These data confirm the well-known increase in fibrous tissue, seen histologically in dystrophic skeletal muscle.

The activity of phosphorylase "a" and "b" was quite reproducible in normal muscle (Tables II and III), phosphorylase "b" being defined as phosphorylase activity in the presence of adenylic acid. In muscle from all but one of the dystrophic patients, phosphorylase activity was reduced greatly, both in terms of wet weight of muscle or of its non-collagen nitrogen content. In both normal and dystrophic muscle, addition of adenylic acid produced an increase of 30 to 40 per cent in phosphorylase activity. Muscle from two patients with severe dystrophy had almost no phosphorylase activity, although half the nitrogen in the muscle samples from them was non-collagen nitrogen.

TABLE II

Phosphorylase "a"

Units per Gm. of wet muscle		Units per Gm. of non-collagenous proteins	
Controls	Dystrophics	Controls	Dystrophics
1435	325	9570	4330
1500	80	8810	1070
1960	483	10800	3710
1360	430	8710	3870
1180	1190	6740	7580
1140	26	6910	346
	262		1820
	164		1500
	120		1200
	385		4000
	297		2380
Mean: 1429±112	360±96	8590±630	2890±544

TABLE III

*Phosphorylase "b" **

Units per Gm. of wet muscle		Units per Gm. of non-collagenous proteins	
Controls	Dystrophics	Controls	Dystrophics
1575	560	10000	7400
2000	142	11800	1900
3150	595	17400	4570
2540	682	16150	6100
1610	1700	8000	10800
1995	70	12000	1000
	638		4400
	420		4000
	245		2450
	470		5000
	420		3360
Mean: 2145±226	540±127	12570±1225	4640±840

* By agreement, the total phosphorylase activity in the presence of adenylic acid is called "phosphorylase b activity."

Aldolase activity (Table IV) varied in normal muscle to a somewhat greater extent than did phosphorylase activity. Nevertheless, this variation did not obscure a definite decrease in aldolase activity in dystrophic muscle, which was, on the average, only a third that of normal, referred to non-collagen nitrogen. Again, in several patients, aldolase activity was nearly absent.

These decreases in phosphorylase and in aldolase activity conceivably might have been due to the presence of an inhibitor in dystrophic muscle. This possibility was eliminated by the results of experiments in which the extracts of dystrophic muscle were incubated with crystalline aldolase

TABLE IV

Aldolase

Units per Gm. of wet muscle		Units per Gm. of dry non-collagenous proteins	
Controls	Dystrophics	Controls	Dystrophics
1.576	0.17	10	2.26
5.45	0.6	32	4.58
4.85	0.745	26.8	6.7
3.4	1.49	21.8	9.4
2.26	0.10	12.8	1.33
2.385	1.45	14.4	10
2.68	0.64	18.6	6
3.88	0.79	23.9	7.9
	1.085		11.0
Mean: 3.31±0.47	0.78±0.16	20±2.75	6.6±1.18

and phosphorylase. No inhibiting effect was noted.

In 36 of 38 patients with muscular dystrophy, aldolase activity of serum was higher than in normal subjects. The mean value was 10 times greater than that of controls. Patients with other neuromuscular disorders and marked wasting of muscle did not show abnormal serum aldolase activity. Details of this study are reported elsewhere (17).

Results of determination of non-heminic iron appear in Table V, with data from earlier studies. Although the iron content was reduced when referred to wet weight of dystrophic muscle, when referred to non-collagen nitrogen, the iron content of dystrophic muscle was exactly the same as that of control muscle. On the contrary, in atrophy of nervous origin, the iron content was increased.

DISCUSSION

Data reported herein demonstrate a real decrease in the activity of two glycolytic enzymes in skeletal muscle of patients with muscular dystrophy and, confirming the observation on two cases by Sibley and Lehninger (14), an increase in the concentration of one of them, aldolase, in serum. It is too soon to say whether these enzymatic defects are the cause of the dystrophy or whether they are simply

TABLE V

Muscle iron

	mg. per 1000 Gm. wet muscle			mg. per 1000 Gm. dry defatted muscle			mg. per 1000 Gm. dry non-collagenous protein		
Muscle	Control	Progressive muscular dystrophy	Atrophy	Control	Progressive muscular dystrophy	Atrophy	Control	Progressive muscular dystrophy	Atrophy
Transversus	25.5	10		125	72		170	133	
	18	14.5		75	72		107	200	
	12	18.4		60	90		66	140	
	30	8		140	39		214	70	
	17.6	15.5		85	77		100	100	
		12.6						87	
		16.4						150	
		8.3						88	
Gluteus maximum	18.5	8.5	20.5	100	50	195			
	17	4	30	75	25	185			
	13.2	15.3	44	68	53	280			
	12.9	9.8	42	65	110	235			
		10.9	32.5		67	210			
		11.5	32		122	170			
		17.5			66				
		11.8			78				
		15.6			90				
		15.6							
Triceps		17	32.5		90	162			280
		8	25		38	135			
		7.5	35		37.5	178			
		17.5	28		112				
		9.3			67				
		12.4			70				
		12			50				
		8.8			63				
		11.7							
Pectoral major		9.6		70					
		24.5		120					
Mean	18.1	12.2	32.15	90	70	195	131	121	

sequelae of a more primary biochemical flaw. Mirski and Wertheimer (18) Humoller, Hatch and McIntyre (19), other investigators (20) and we (unpublished observations) have demonstrated decreased muscle phosphorylase activity following transsection of the sciatic nerve, and we have observed it also in muscle made atrophic by tenotomy. Fischer (21) similarly described a fall in muscle aldolase activity following sciatic section. In all these cases the decrease in enzyme activity was secondary to the atrophy and was detected by reference to fresh weight of tissue. It may also be noted that decrease in enzyme activity appeared late in these situations when atrophy was well-established, although it cannot be implied that the decreases in dystrophic muscle represent an early biochemical lesion.

Nor can it be stated whether or not the decrease in aldolase and phosphorylase activity is unique. It is entirely possible that more widespread disturbance of glycolytic activity, and even of other enzymes may occur in muscular dystrophy.

SUMMARY

1. Collagen and elastin content of dystrophic muscle was increased.

2. In muscle obtained by biopsy from patients with progressive muscular dystrophy there was a decrease in the activity of phosphorylase and of aldolase. This decrease was demonstrable either when the values were expressed in terms of the wet weight of the sample or in terms of its remaining muscle fibrillar protein, non-collagen nitrogen.

3. Aldolase activity of serum in dystrophic patients was increased many times above the normal value.

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THE VOLUME AND DISTRIBUTION OF BLOOD IN THE HUMAN LEG MEASURED *IN VIVO*. I. THE EFFECTS OF GRADED EXTERNAL PRESSURE [1, 2]

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(Submitted for publication July 1, 1953; accepted January 27, 1954)

Venous pressure-volume curves obtained by venous congestion may be useful for the study of venous tone in human extremities. However, such pressure-volume curves should be measured from a constant reference point or baseline of venous volume and *effective* venous pressure.

The purpose of this paper is to show that such a constant baseline is obtained when an external pressure equal to or greater than the natural local venous pressure is applied to the leg. This has been demonstrated by measuring the volume of blood in the human leg, *in vivo*, at graded external pressures. The data to be presented are not only of physiological interest, but also have a practical bearing on the use of elastic compression to accelerate the linear velocity of blood flow in the leg veins for the prevention of thromboembolic complications in hospitalized patients (1–3).

There have been no quantitative measurements of the volume and distribution of blood in the vessels of the leg, *in vivo*, with the circulation under normal conditions of rest. Several investigators have reported a few measurements of the blood volume of the leg, using the Evans blue dye (4) or the carbon monoxide (5) method, but their results do not give accurate information about the distribution of blood in the vessels of the leg. Neither method is adaptable for our purpose.

The method employed in the present study is to express the blood from the leg in a water-filled plethysmograph and then to record the increase in leg volume which occurred when the circulation was restored. This was repeated over a wide range of external water pressures applied to the leg. The rationale of the method depends upon the assumption that the volume of a blood vessel varies directly with the effective intravascular pressure, if other factors are controlled. This assumption has been thoroughly validated by Ryder, Molle, and Ferris using isolated veins (6).

[1] This work was supported by a grant from the Life Insurance Medical Research Fund.

[2] This paper was presented, in part, at a meeting of the New England Cardiovascular Society on February 19, 1951.

APPARATUS

The water plethysmograph (Figure 1) described by Wilkins and Eichna (7) was modified at the open ends by replacing the rubber diaphragms (formerly cemented to the skin) with a loose-fitting, thin rubber sleeve (8). The ends of the sleeve were everted and permanently sealed to the flanges at each end of the plethysmograph. The spaces between the leg and the ends of the plethysmograph were closed off by loose-fitting masonite plates which were held firmly against the flange by brass plates (7). When the box was filled with water the hydrostatic pressure forced the loose sleeve in folds against the skin (which had been powdered with talc) and against the inner surfaces of the masonite plates.

A highly satisfactory pressure gasket was made from relatively inelastic, tough neoprene rubber, 1/64 inch thick (Figure 1, Insert). Such a gasket was placed at each end of the leg segment between the thin rubber sleeve and the skin and inner side of the masonite plates. This prevented ballooning of the sleeve between the skin and the inner edge of the masonite plates when high pressures were applied to the water in the plethysmograph. External metal struts (not shown in Figure 1) were attached to the ends of the apparatus so that threaded bolts could be tightened against the outer side of the masonite plates near the free inner edge. This arrangement limited outward movement of the plates under high pressure.

The plethysmograph was connected by rubber tubing to a 100 cc. burette, which was supported by clamps so that it could be raised and lowered rapidly. The volume of water in the burette-plethysmograph system (Figure 1) was adjusted by opening stopcock (C) to tube (D) which led to a water reservoir. Water was allowed to run into the reservoir by gravity or was transferred from the reservoir to the burette by a rubber pressure bulb. The burette-plethysmograph system was pressurized through tube (A) connected to the top of the burette by turning a stopcock to a 20-liter air pressure reservoir.

A narrow blood pressure cuff (H), 8 cm. wide, was placed on the ankle close to the distal end of the plethys-

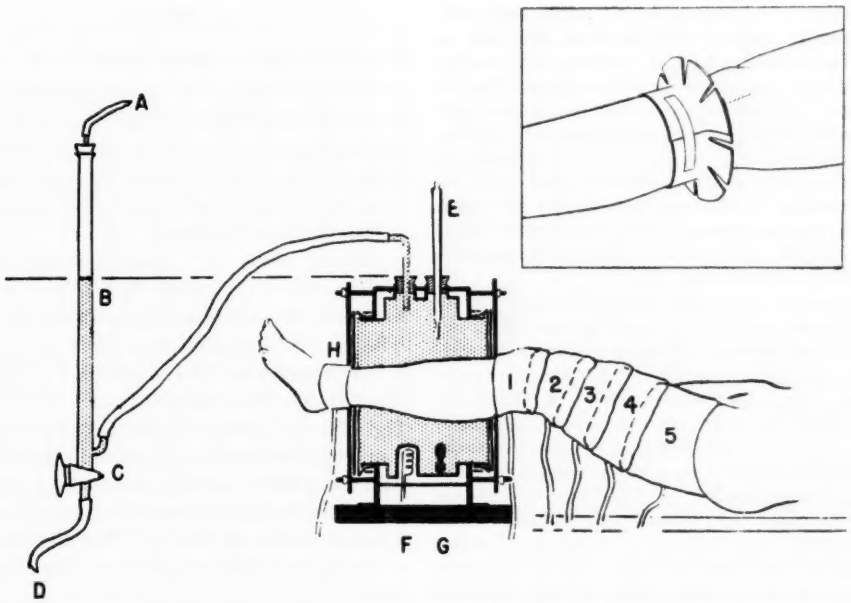

FIG. 1. THE BURETTE-PLETHYSMOGRAPH APPARATUS ON THE LEG OF A SUPINE SUBJECT

"F" is an electric water heater, "G" is a propeller, "E" is a thermometer. The posterior surface of the leg is at heart level. See Text.

Insert: Diagram of neoprene rubber pressure gasket below the knee. The overlapping ends of the loose-fitting gasket are secured with adhesive tape.

mograph. Five cuffs, 14 cm. wide, were placed on the knee and thigh, the lower cuff overlapping the next higher cuff. Two cuffs were used in positions 1, 2, and 5, with the rubber bags on opposite sides of the extremity for more efficient transmission of pressure. All these cuffs were connected through stopcocks to air pressure reservoirs. The thigh cuffs were firmly supported with adhesive tape. Cuffs 1, 2, 3, and 4 were taped to each other to prevent slipping and bulging. This greatly reduced the discomfort at high cuff pressures.

PROCEDURE

The subjects were healthy young men. One group of experiments was done with the subject supine and the posterior surface of the leg segment in the plethysmograph at heart level, measured 10 cm. anterior to the back. Another group of experiments was done with the upper half of the body uptilted 13 to 18 degrees, and the leg under study slightly lowered, to increase the local venous pressure in the leg. In these experiments heart level was estimated from the phlebostatic level (9). In both groups of experiments a rolled blanket was placed under the thigh and sandbags under the foot so that the extremity was comfortable and relaxed, with the knee only slightly higher than the ankle. The temperature of the water in the burette-plethysmograph and the water reservoir was 32° C.

The ankle cuff was inflated to a pressure of 260 mm. Hg to prevent circulation distal to the plethysmograph. One minute later the air pressure in the burette-plethysmograph system was raised to 250 mm. Hg to express the blood from the leg toward the heart. Water was pumped into the burette from the reservoir as water flowed from the burette into the plethysmograph. This high pressure tended to force the apparatus toward the ankle but such movement was limited by the ankle cuff which exerted counterpressure against the masonite plates. Two minutes later cuff 1 was inflated to 250 mm. Hg and at 20-second intervals cuffs 2 and 3 were similarly inflated. Then the air pressure in the burette plethysmograph system was reduced to atmospheric and cuffs 4 and 5 were inflated at 20-second intervals to a pressure of 250 mm. Hg. This completed the expression of blood proximally from the leg and thigh. The pressure in cuffs 1, 2, 3, and 4 was then reduced to atmospheric, so that only the highest cuff, 5, prevented reentry of blood into the extremity.

The external water pressure on the leg was then adjusted at the desired level as measured in the burette in mm. Hg with reference to heart level. The subject was urged to relax and the pressure was abruptly released from the highest cuff, 5, at zero time. Blood rushed into

the empty vessels very rapidly due to reactive hyperemia, which gradually subsided until the blood flow had returned to the normal resting level. At first, blood entering the leg displaced water from the plethysmograph into the burette; later, as reactive hyperemia subsided, water flowed back from the burette into the plethysmograph. During this time the burette was rapidly lowered, then raised, so that the water level in it and water pressure on the leg remained constant. It was important that the water pressure on the leg was constant during the observations to prevent errors due to variations in volume of the apparatus with variations of pressure.

Burette readings were taken every 15 seconds for five to eight minutes, until the leg volume did not change significantly for two minutes, indicating that reactive hyperemia was over and the vascular volume had returned to the resting value. The vascular volume of the leg was calculated from the burette readings and the leg volume. The leg volume was calculated from the known capacity of the plethysmograph and the volume of water it contained with the leg segment in it. The initial level of the water in the burette was a few centimeters above the top of the plethysmograph in both groups of experiments. The procedure was repeated numerous times in each experiment on each subject, over a range of external water pressures on the leg up to 60 mm. Hg above the initial water pressure. The water pressure was usually raised by increments of 5 or 10 mm. Hg.

The assumption that all, or almost all, of the blood in the leg was expressed by an external pressure of 250 mm. Hg will be considered in the Discussion.

RESULTS

Three experiments on uptilted subjects

The data obtained in three consecutive experiments on three different subjects studied with the trunk in the (13° to 18°) uptilted position were similar (Figure 4). Therefore, only one experiment will be discussed in detail and average data will be discussed for the group.

Detailed data. The vascular volume of the leg in subject D. H. from the time blood was allowed to reenter the empty vessels is shown in Figure 2. The number opposite each curve is the external water pressure on the leg in mm. Hg (referred to heart level), which ranged from − 5 to + 55 mm. Hg. At the lowest external water pressure (− 5 mm. Hg), the level of the water in the burette was 21.6 cm. (15.9 mm. Hg) above the posterior surface of the leg and 6.7 cm. (4.9 mm. Hg) above the anterior surface of the leg. The posterior surface of the leg was 28.4 cm. (20.8 mm. Hg) below heart level

During the first minute after the circulation was restored, the vascular volume reached a peak which varied inversely with the external water pressure. This early peak volume was associated with the rapid blood flow of reactive hyperemia and was

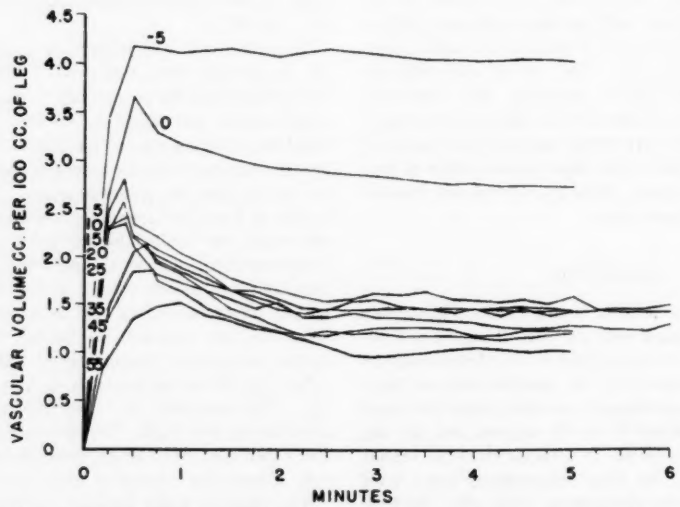

FIG. 2. TIME-VASCULAR VOLUME CURVES IN SUBJECT D. H. AFTER BLOOD WAS ALLOWED TO RE-ENTER THE BLOODLESS EXTREMITY

The number opposite each curve is the external water pressure (mm. Hg) referred to heart level, applied to the leg during this period. See Text.

called the peak reactive hyperemia vascular volume, or P.R.H.V.V. Then, at each external water pressure the vascular volume gradually decreased and leveled off as the blood flow subsided to the resting value. The average of the vascular volumes recorded during the last two minutes of each curve when only minor variations in volume occurred was designated the resting vascular volume, R.V.V.

The P.R.H.V.V. and the R.V.V. at each external water pressure for subject D. H. are shown in Figure 3. The effect of increasing the external water pressure on the P.R.H.V.V. was similar to that described by McLennan, McLennan, and Landis, who studied the forearm (10). The interpretation of the factors which determined this curve is complex and not immediately relevant to the present paper. However, we do not subscribe to the explanation which has been suggested (10).

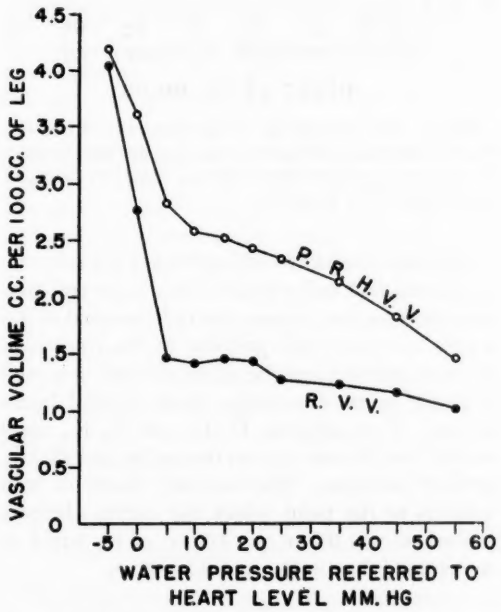

FIG. 3. THE EFFECT OF INCREASING THE EXTERNAL WATER PRESSURE APPLIED TO THE LEG ON THE PEAK REACTIVE HYPEREMIA VASCULAR VOLUME AND THE RESTING VASCULAR VOLUME IN SUBJECT D. H.

The P. R. H. V. V. occurred during the first minute after blood was allowed to re-enter the extremity (Figure 2) and the R. V. V. was the average of the last two minutes of each curve (Figure 2) when there were only minor variations in vascular volume.

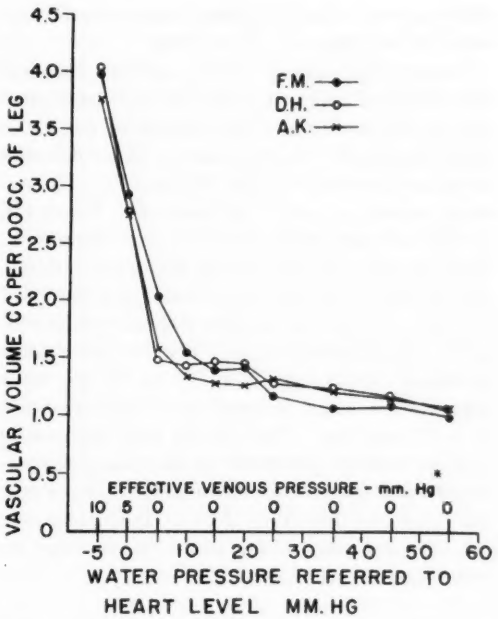

FIG. 4. THE EFFECT OF INCREASING THE EXTERNAL WATER PRESSURE APPLIED TO THE LEG ON THE RESTING VASCULAR VOLUME OF THREE YOUNG, HEALTHY MEN, WITH THE TRUNK UPTILTED 13 TO 18°

An increase in water pressure of only 10 mm. Hg (from − 5 to + 5 mm. Hg) in two subjects (D. H. and A. K.) and 15 mm. Hg in the third (F. M.) caused a large decrease in vascular volume.

* See Discussion. The effective venous pressure scale refers to subjects D. H. and A. K. only.

The primary concern was to determine the effect of increasing the external water pressure on the *resting* vascular volume. The vascular volume was 4.0 cc. per 100 cc. of leg when the external water pressure was − 5 mm. Hg referred to heart level; it decreased to 2.7 cc. when the external water pressure was increased (by 5 mm. Hg) to 0 mm. Hg and to 1.5 cc. when the external water pressure was further increased to + 5 mm. Hg. After this, with further increases in external pressure the resting vascular volume abruptly leveled off. Thus, when the external water pressure on the leg was increased by only 10 mm. Hg (from − 5 to + 5 mm. Hg), a large decrease in vascular volume occurred, from 4.0 cc. to 1.5 cc. But, when the external water pressure was increased an additional 50 mm. Hg (from + 5 to + 55 mm. Hg),

there was only a small further decrease in the vascular volume, from 1.5 cc. to 1.0 cc.

Average data in the three uptilted subjects. The effects of increasing the external water pressure on the resting vascular volume of the leg in three consecutive experiments on three different subjects were very similar (Figure 4). The average volume at a water pressure of − 5 mm. Hg referred to heart level was 3.9 cc. An increase in water pressure of only 10 mm. Hg in two subjects (D. H. and A. K.) and of 15 mm. Hg in the third (F. M.) reduced the vascular volume in each subject to approximately 1.5 cc. The vascular volume decreased only 0.5 cc., from 1.5 to 1.0 cc., when the external water pressure was further raised to + 55 mm. Hg. Thus, of the total decrease in vascular volume produced by increasing the external water pressure by 60 mm. Hg, 82.5 per cent was due to the first 10 or 15 mm. Hg increase, and only 17.5 per cent was due to the further large increase in pressure.

Four experiments on supine subjects

A second group of four experiments was done with the subjects supine and with the posterior surface of the horizontal leg segment at approximately heart level (Figure 5). (Two of the four subjects, D. H. and A. K., were also studied in the slightly uptilted position.) The lowest water pressures applied to the leg were 11.5 to 13.2 mm. Hg above heart level. The initial resting vascular volumes were 1.8 cc., 1.7 cc., 2.0 cc., and 2.3 cc. per 100 cc. of leg. When the external water pressure was increased the vascular volumes decreased and leveled off at pressures of 20, 17.5, 16.7, and 16.5 mm. Hg corresponding to vascular volumes of 1.2, 1.1, 1.2, and 1.3 cc. Further increases in the external water pressure to 55 or 65 mm. Hg caused only minor decreases or variations in vascular volume.

These experiments on supine subjects provide additional evidence that low external water pressures applied to the leg reduce the vascular volume which abruptly levels off and is little affected by further large increases in external pressure. The vascular volumes were more irregular than in the slightly uptilted subjects. It was assumed that this was due to more mechanical movement of the extremity in the supine position.

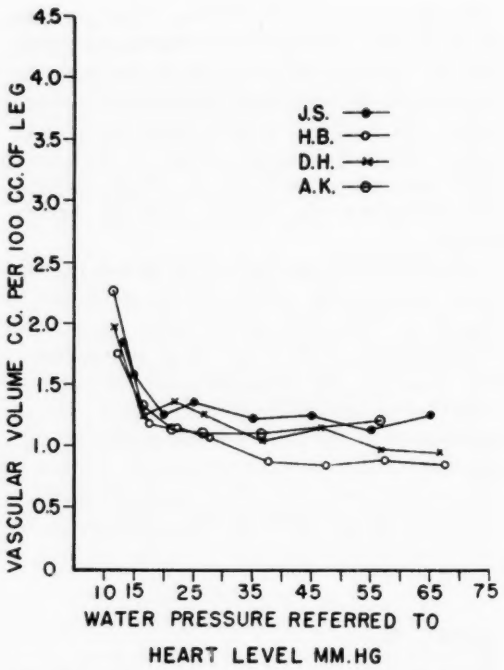

FIG. 5. THE EFFECT OF INCREASING THE EXTERNAL WATER PRESSURE APPLIED TO THE LEG ON THE RESTING VASCULAR VOLUME OF FOUR YOUNG, HEALTHY MEN, IN THE SUPINE (0°) POSITION

The data obtained in both groups of experiments were consistent and reproducible. In several subjects the vascular volume was redetermined at the lowest external water pressure at the conclusion of the experiment and the values checked very well with the initial observation made several hours earlier. Two subjects, D. H. and A. K., were studied on different days in the supine and slightly uptilted positions. The vascular volume of both subjects at the point where the curves abruptly leveled off was 0.3 cc. per 100 cc. of leg larger in the uptilted than in the supine position.

DISCUSSION

Effective venous pressure

The effective venous pressure is defined as the internal pressure minus the external pressure. The internal pressure which tends to distend a vein is due to the lateral pressure of the blood flowing through it. The lateral pressure is not sig-

nificantly different from the venous pressure as ordinarily measured and is opposed by the external pressure which tends to collapse the vein. The external pressure is the tissue pressure, usually about 3 mm. Hg (11). In our experiments the leg was immersed in water and therefore the tissue pressure was increased by the external water pressure. Obviously, an increase in the external water pressure applied to the leg would decrease the effective venous pressure and therefore would decrease the volume of the veins. These considerations apply as well to the venules, capillaries, arterioles, and arteries.

Normally there is a hydrostatic gradient of pressure in the blood vessels of the horizontal leg, when the extremity is at or below heart level. When the leg is immersed in water in a plethysmograph the external pressure of the water on the leg also has a hydrostatic gradient which counterbalances the hydrostatic vascular pressure (if one ignores the slight error due to the greater specific gravity of blood). Therefore, the effective vascular pressures in an immersed limb do not have a hydrostatic gradient and are nearly the same in similar blood vessels (all the veins or all the arteries) whether they are near the anterior or the posterior surface of the leg. Furthermore, the effective vascular pressures in such an immersed limb are not altered by changing the position of the limb (at or below heart level) but are determined by the local venous pressure referred to heart level and the external water pressure referred to heart level.

In the discussion which follows, reference will be made mostly to data obtained in the slightly uptilted subjects. However, the comments apply also to the supine subjects.

The constant baseline of venous volume and effective venous pressure

The large decrease in vascular volume produced by the first 10 or 15 mm. Hg increase in external water pressure (Figure 4) must have been due almost entirely to a decrease in the effective pressure and volume of the lowest pressure vessels, the veins (and venules). For, if there had been a significant decrease in the caliber of the capillaries and arterioles there would also have been a significant decrease in the volume flow of blood through the leg segment, since the resistance varies inversely with the fourth power of the diameter of a tube (Poiseuille's law). We have measured the blood flow before, during, and after comparable changes in effective venous pressure produced by such small increases in external water pressure on the leg and observed no significant change (12).

There was abrupt leveling of the vascular volume curves of the leg at an external water pressure of + 5 or 10 mm. Hg above heart level (Figure 4), corresponding to an estimated effective venous pressure near zero. These facts are additional evidence that the preceding large decrease in vascular volume occurred almost entirely in the veins. One can assume that when the water level was raised until the external pressure on the leg became equal to the initial local venous pressure, the effective venous pressure did not become zero, but slightly greater than zero. For at zero the veins at first may have been completely collapsed but, as arterial inflow continued, the pressure in the minute vessels and veins must have risen until the venous pressure was slightly greater than the external pressure (effective venous pressure slightly greater than zero) at which point the veins reopened and venous outflow resumed. This would also occur at all external water pressures greater than the initial local venous pressure, up to diastolic arterial pressure.

Thus, when the external water pressure was equal to or greater than the initial local venous pressure, the effective venous pressure was constant, slightly greater than zero. This is the minimum effective venous pressure possible when the blood is circulating. The venous volume corresponding to this minimum effective venous pressure also must have been constant and is called the "baseline venous volume." These conclusions are consistent with the observations of Ryder, Molle, and Ferris that the volume of an isolated vein *in vitro* is determined by the effective venous pressure regardless of the absolute value of the external pressure (6). We have obtained experimental evidence *in vivo* that when the external pressure on the leg is equal to or greater than the local venous pressure the venous volume is virtually constant at the baseline value (11). The venules probably constitute a minor portion of the vascular volume which we have ascribed to the veins (11).

The importance of the effective venous pressure as the determinant of vascular volume.

It may now be inferred that the average vascular volume of 3.9 cc. at an external water pressure of − 5 mm. Hg referred to heart level was measured at an effective venous pressure of approximately 10 mm. Hg in two subjects, D. H. and A. K. (Figure 4), and 15 mm. Hg in the third, F. M. Increasing the water pressure 10 or 15 mm. Hg reduced the effective venous pressure to slightly greater than zero and caused a large decrease in vascular volume of 2.4 cc., from 3.9 cc. to 1.5 cc., which occurred in the veins. The vascular volume of 1.5 cc. included the baseline venous volume and the "normal" volume of the capillaries, arterioles and arteries. The small decrease in vascular volume of only 0.5 cc. from 1.5 cc. to 1.0 cc., produced by raising the external water pressure an additional 45 or 50 mm. Hg, was attributed to decreases in the effective pressures of the capillaries, arterioles and arteries, the effective pressure of the veins remaining slightly greater than zero and the venous volume constant, at the baseline value.

At the highest external water pressure, + 55 mm. Hg referred to heart level, the arteriovenous pressure gradient was considerably decreased. The effective arterial pressure was 60 mm. Hg less than at the start of the experiments and the effective arteriolar and capillary pressures quite low, particularly the latter. The small decrease in vascular volume which occurred, 0.5 cc., as a result of the large decreases in effective pressure in the capillaries, arterioles and arteries is further evidence that the combined volume of these vessels is small compared to the veins.

Thus it appears that the volume and distribution of blood in the leg at rest are essentially functions of the effective venous pressure. The vascular volume is always small, 1.5 cc. per 100 cc. of leg or less, when the effective venous pressure is at the physiological minimum (slightly greater than zero). Large increases in volume occur as a result of modest increases in the effective venous pressure above the minimum value. Although the quantitative relationship between effective venous pressure and venous volume unquestionably varies among the different organs of the body, it is likely that the vascular volume of any organ is a function mainly of the effective venous pressure.

The venous pressure-volume curve

The concepts elaborated in the foregoing discussion suggest that venous pressure-volume curves measured from the baseline venous volume at an effective venous pressure slightly greater than zero as a point of reference should be physiologically valid. Increases in venous volume produced by venous congestion could then be related to known effective venous pressures. A relatively simple method for determining the venous pressure-volume curve utilizing these principles has been developed and will be presented in another paper (11).

Is all the blood expressed from the leg by an external pressure of 250 mm. Hg?

It is at present impossible to answer this question exactly. However, the main thesis of this paper is valid whether or not all the blood was expressed from the leg, provided that a constant fraction was always removed. The following facts indicate that such was the case. The same vascular volume was obtained in several experiments whether expressing pressures of 200, 250 or 300 mm. Hg had been applied to the plethysmograph. Furthermore, the same vascular volume was obtained repeatedly in the same subject when the same low external pressure was used while blood reentered the limb. Finally, there was good agreement among the data obtained in different subjects.

Other considerations justify the assumption that the fraction of the limb blood volume which may not have been expressed was small.

1. When a lucite (transparent) plethysmograph was filled with water, it could be seen that little if any air was trapped in the irregular, horizontal folds of the loose rubber sleeve which surrounded the leg. This seemed a reasonable analogy with what occurred in the blood vessels.

2. The vascular volume of the leg as determined in our experiments is of the same order as the values obtained by other workers who measured the blood volume by the Evans Blue dye (4) and the carbon monoxide (5) methods. There were great variations among the results obtained by these workers because of the large errors inherent in the methods employed and *because the veins in the legs of their subjects were at different and un-*

known effective venous pressures. Ebert and Stead (4) found the average blood volume in one upper and the two lower extremities of five subjects to be 900 cc. If one assumes that the average volume of each lower extremity was 13 liters (5) and of one arm 4 liters, then the average blood volume in the extremities of these supine subjects was 3.00 cc. per 100 cc. of limb. This may be compared with our data by selecting the vascular volume in Figure 4 corresponding to the average effective venous pressure in the leg of a supine subject. The average venous pressure in the leg of a supine person referred to heart level (about 10 cm. anterior to the bed) is 13 cm. H_2O (13). Since the average male leg is about 13.5 cm. thick the bulk of the leg is below heart level and the average *local* venous pressure would be somewhat higher, about 15 cm. H_2O or 11 mm. Hg. Subtracting 3 mm. Hg for the average tissue pressure in the leg (11), the average *effective* venous pressure would be 8 mm. Hg. Accordingly, the vascular volume of the leg of a supine subject without a plethysmograph on the extremity would be approximately 3.5 cc. per 100 cc. of leg (Figure 4). This compares favorably with the value of 3.00 cc. derived above.

In summary, it appears that almost all, if not all, the blood was expressed from the leg in our experiments when the pressure in the plethysmograph was raised to 250 mm. Hg. In any case, the fraction of the blood volume which may have remained in the leg was constant and relatively small.

The efficient transmission of external water pressure to the leg

If *low* external water pressures were not efficiently transmitted to almost the entire leg segment in the plethysmograph one would expect that falsely high values for vascular volume might be obtained due to the accumulation of blood in places where the effective vascular pressures were little affected by the low external pressure, namely at the thick proximal end of the leg segment (14). If this were true, then when the external water pressure was raised 5 or 10 mm. Hg the transmission of this pressure to the proximal part of the leg might become much more efficient, resulting in marked decreases in vascular volume such as those observed.

However, comparison of the experiments done in the uptilted and supine subjects indicates that if such an artefact existed it was not important. The lowest water pressure used, when referred to the surface of the leg, was practically the same in both groups of experiments, but the average vascular volumes were distinctly different: 3.9 cc. for the uptilted (Figure 4) and 1.9 cc. for the supine (Figure 5) subjects. The larger vascular volume in the uptilted subjects was due to the higher effective venous pressure in the leg in this position resulting from the fact that the leg was below heart level, whereas the legs of the supine subjects were at heart level. Furthermore, it required only about 5 mm. Hg additional water pressure in the supine subjects to reduce the effective venous pressure to slightly greater than zero after which the vascular volume remained almost constant, whereas in the uptilted subjects it required 10 or 15 mm. Hg additional water pressure to achieve the same result. Since the vascular volume in each case was dependent on the effective venous pressure rather than on the absolute value of the external water pressure referred to the surface of the leg, one must conclude that the water pressure was efficiently transmitted.

The effect of external pressure on the interstitial fluid volume

Landis and Gibbon showed that when a pressure of 200 mm. Hg was applied to the forearm for two minutes the volume of interstitial fluid expressed was insignificant (15). This probably also applies to the leg at a pressure of 250 mm. Hg for two minutes. The resting vascular volume was measured at external water pressures up to 60 mm. Hg five to eight minutes after blood flow resumed. During this period no more, and probably less, interstitial fluid could have reaccumulated than was expressed by the high pressure of 250 mm. Hg. Therefore, the values obtained for the resting vascular volume measured at external pressures up to 60 mm. Hg included only insignificant amounts of interstitial fluid.

SUMMARY AND CONCLUSIONS

The leg was rendered as bloodless as possible by pressurizing it in a water-filled plethysmograph at a suprasystolic pressure. Blood was prevented from reentering the leg by a cuff at suprasystolic

pressure proximal to the plethysmograph while the water pressure in the plethysmograph was readjusted to an exact (low) level. The circulation was then restored and the volume of blood that entered the leg at this external pressure was measured after reactive hyperemia had subsided. Such measurements were repeated over a wide range of external water pressures.

Small increases in external water pressure produced large decreases in the resting vascular volume which leveled off abruptly when the external pressure had risen to equal the venous (or venular) pressure. At that point the effective venous pressure was reduced to slightly greater than zero and the venous volume was at the "baseline value." The large decrease in vascular volume was attributed to the reduction in the effective venous (and venular) pressure. Further large increases in the external water pressure caused only small decreases in the vascular volume, attributed to reductions in the effective pressures of the capillaries, arterioles, and arteries.

It was concluded that:

1. A constant baseline of venous volume at an effective pressure slightly greater than zero is obtained when an external pressure equal to or greater than the natural local venous pressure is applied to the leg.

2. Changes in vascular volume produced by changes in external pressure are due almost entirely to changes in effective venous (and venular) pressure.

3. The combined volume of the capillaries, arterioles, and arteries is small regardless of the effective pressures in these vessels.

4. The volume and distribution of blood in the leg at rest are essentially functions of the effective venous pressure.

ACKNOWLEDGMENT

The authors wish to thank Dr. Robert W. Wilkins for his constant encouragement and helpful criticisms.

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THE EFFECT OF POTASSIUM CHLORIDE ON HYPONATREMIA [1]

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(Submitted for publication August 31, 1953; accepted February 10, 1954)

In cardiac edema as well as in various other states characterized by excessive retention of fluid there is observed frequently an abnormally low concentration of sodium in the serum and extracellular fluid (1, 2). Rigorous sodium restriction, mercurial diuretics, and cation exchange resins may exaggerate or produce this tendency to hypotonicity. Sodium administration often enhances accumulation of fluid, without increasing its tonicity, and hypertonic sodium chloride, though effective at times, may be neither beneficial nor safe (3).

Because of the shortcomings of these various therapies and because hyponatremia *per se* may play a role in the production of adverse symptoms, it seemed desirable to search for another diuretic agent which might promote the loss of excessive body water without aggravating disturbances in sodium metabolism. It was decided, therefore, to observe the effects of potassium salt in hyponatremia, not only because of its possible action as a diuretic agent but also because of its relation to sodium metabolism.

Potassium salts have long been considered a useful diuretic agent. "Salt of niter" (potassium nitrate) was recommended for the treatment of dropsy in 1679 by Thomas Willis (4). In 1920, Magnus-Levy (5) and Blum (6) showed that potassium chloride could be administered safely by mouth in relatively large doses in various edematous states and that it often produced a diuresis. Similar results have been obtained by others (7–9). Potassium salts have produced diuresis without excessive loss of sodium, acting apparently through suppression of renal tubular secretion of hydrogen ions (10) and possibly as a result of osmotic activity of electrolytes of tubular urine.

In addition to the renal effects of potassium salts, it was thought possible that their administration might favorably influence disturbances in sodium metabolism manifested by pathologic distribution of sodium and potassium within the body. Two recent studies have served to emphasize this important relationship between sodium and potassium. *In vivo*, with potassium depletion there is a movement of sodium ions into cells and an associated extracellular alkalosis. This intracellular sodium can then be mobilized by potassium administration (11). *In vitro* it has been shown that potassium transport is dependent upon energy of aerobic oxidation. If the cell is injured or metabolically inhibited potassium fails to accumulate and is replaced by an influx of sodium and water (12).

Accordingly, potassium chloride (KCl) was given to an edematous, hyponatremic cardiac patient with the hope of effecting water diuresis. The procedure produced a reduced urine volume. Moreover, a striking increase in the serum sodium was observed at a time when there was no change in the external balance of sodium. This phenomenon seemed of sufficient interest to warrant further study. The observed and derived transfers of sodium and potassium were, therefore, studied by the balance method following KCl administration in six patients with and without edema.

METHOD

Patient selection. Six patients, ranging in age from 22 to 65 years, were studied on the medical or metabolic wards of the Presbyterian Hospital. The criteria for selection included hyponatremia (serum sodium less than 126 mEq. per liter) and the absence of advanced renal disease (blood urea nitrogen less than 30 mg. per cent, except for one value of 39 on one occasion in a single patient). One subject each with the following conditions was studied: rheumatic heart disease with edema, Laënnec's cirrhosis with ascites, tetralogy of Fallot with edema, tuberculous meningitis, and unclassified collagen disease with accelerated hypertension and proteinuria. The latter two patients had no detectable edema. In addition, one patient was studied with edema due to cor pulmonale but without significant hyponatremia.

[1] This work has been aided by a grant from the National Heart Institute (USPHS).

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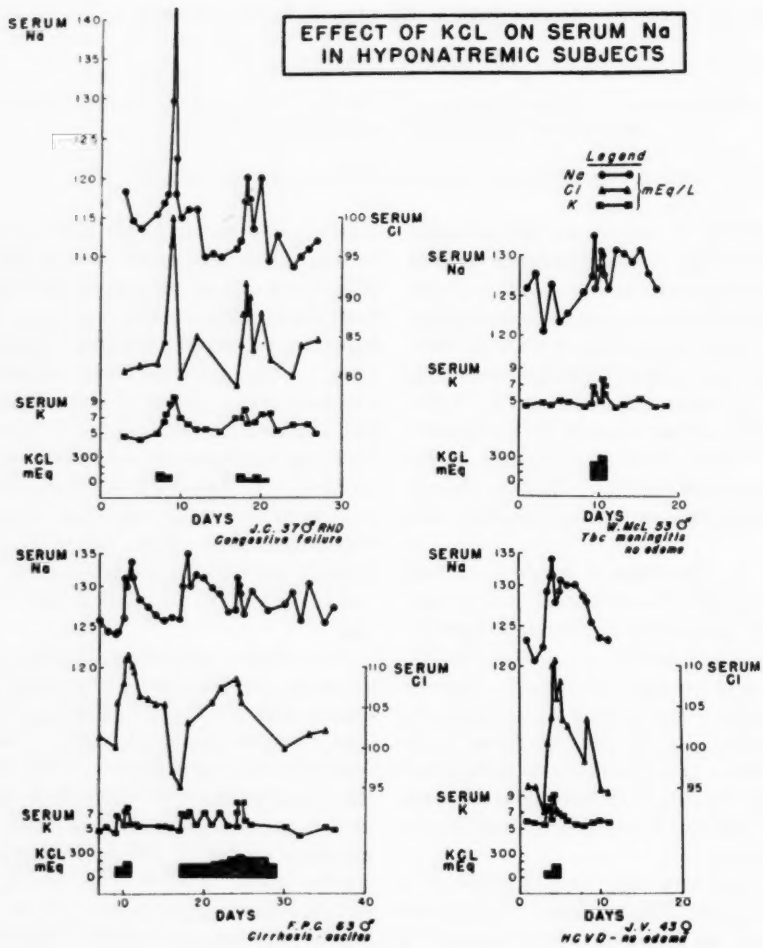

FIGURE 1

Procedure. Four of the patients were transferred to the metabolism ward, where they were treated throughout the study with bed-rest and sedatives. No diuretic was given, but digitalis therapy was maintained in constant dosage. Weights were measured in the fasting state. The patients were maintained on constant amounts of distilled water and on a constant weighed, low sodium diet, the nature and quantity of which were selected by the patient during a preliminary period.

The balance studies were divided into periods of from three to six days during which the daily diet was kept constant by weight from identical food lots. Daily diets were analyzed in duplicate periodically for sodium and potassium content and the composition of any vomitus or uneaten residue was determined. Urine was collected during consecutive 24 hour periods (8 a.m. to 8 a.m.), measured for volume, and analyzed daily for sodium, potassium, and chloride. All stools were pooled for each period and analyzed similarly.

Venous blood was drawn without the application of a tourniquet when possible and was delivered under oil for CO_2 determinations. Blood specimens were usually obtained daily in control periods and as often as six times per day during periods of KCl administration. Blood was allowed to clot for 45 minutes and then separated. Serum was then analyzed for sodium, potassium, chloride, CO_2, urea, and total protein. In two patients, balance studies were not done and serum electrolytes only were observed. A 20 per cent solution of KCl was administered orally in amounts ranging from 54 to 295 mEq. (4 to 22 grams) per day and given in divided doses, three to four times a day. Serum potassium levels were followed closely after each dose and serial electrocardiograms were taken.

Analytical methods. Sodium and potassium were determined with a barrier layer photo cell flame photometer (lithium internal standard) having an accuracy of ± 1 per cent (13). Chloride was determined by the method of Schales and Schales (14) or by a modified Volhard titration. Serum carbon dioxide content was measured with the Van Slyke manometric apparatus. Serum urea nitrogen was determined by direct nesslerization according to the method of Gentzkow (15). The hematocrit was determined by the Wintrobe method, and serum protein by a specific gravity gradient tube by Lowry's modification of the micro-method of Linderstrøm-Lang (16). The exchangeable body sodium (24-hour) as well as the 4-hour sodium space was determined employing Na^{24} after the method of Forbes and Perley (17).

Analyses of diet and stool were made in triplicate. These specimens were homogenized and made up to a known volume with distilled water. Aliquots for sodium and potassium determinations were digested until clear with equal volumes of concentrated nitric acid on an electric hot plate, filtered, and made to volume with lithium for analysis by flame photometer

Calculations. In the balance studies the total balance for sodium and potassium represents the difference between intake (calculated as the sum of diet and medications) and output (the sum of losses in the urine and stool). Because of the nature of the observed changes, no corrections were made for electrolytes lost by bloodletting, and the potassium balance was not corrected for changes in nitrogen balance. No studies were carried out in the summer months.

In considering possible changes in the distribution of sodium and potassium between the extra- and intracellular compartments, the intracellular balance was calculated as the difference between the measured external balance and an estimate of the extracellular space of 20 per cent of body weight. This latter calculation is not essential to these studies but is used as a means of depicting the type and direction of some of the changes. Since most of the patients were edematous, the assumed extracellular space is probably considerably less than the true value, and the postulated shifts of electrolyte are therefore estimated conservatively. For example, in two of the patients, J. C. and F. P. C., the four-hour Na^{24} space was found to be 36 and 39 per cent of body weight, respectively. However, essentially the same conclusions resulted from calculations based on larger initial extracellular volumes.

TABLE I

Dosage schedules of KCl and observed effects on serum potassium

Pt.* Date	Total dose of KCl	Duration of the dosage period	Serum potassium		Time for return to control level (after last dose)
			Control†	Observed peak	
	mEq.	*days*	*mEq./L.*	*mEq./L.*	*hrs.*
J. C. 3/7	187	2	4.5	9.5	120
J. C. 3/18	107	1.5	5.7	7.9	72
F. P. C. 4/9	322	2	4.7	7.5	26
F. P. C. 4/17	322	2	5.1	7.1	24
F. S. 12/6	121	1.5	4.5	5.7	48
A. M. 1/6	563	5	4.7	6.5	>60
A. M. 1/20	161	2	5.0	5.6	48
J. V. 4/14	241	1.5	5.7	9.2	40
W. Mc. 5/5	523	2	4.5	7.3	18

* All patients were studied while on constant low sodium diet containing from 7 to 15 mEq. of sodium except for Pt. A. M. who was studied while on constant low sodium and again (1/20) on constant higher intake of 68 mEq. of sodium daily.

† Controls = average of three determinations.

RESULTS

The results of these studies are summarized in Figure 1 and Tables I, II, and III. In Table IV more complete metabolic data from one patient are presented.

The first patient (J. C., Figure 1 and Tables I, II, and III) was a 37-year-old man with long standing rheumatic heart disease. He had had atrial fibrillation for at least five years and, despite vigorous treatment including injections of mercuhydrin two or three times weekly, had been in chronic, right-sided failure for two years. This patient had the following serum electrolyte values: sodium 128 mEq per L., potassium 5.3 mEq. per L., chloride 96 mEq. per L., and CO_2 content 29 mm. per L. After eight days of Carbo-Resin (Lilly), 60 Gm. daily, there was a further gain in weight, the patient became weak and fatigued, and the serum sodium fell to 114 mEq. per L. and the chloride to 81 mEq.per L. The serum CO_2 content did not change significantly. These changes could not be accounted for by the small amounts of sodium and chloride excreted and probably represented, therefore, either a relative hemodilution, or a transfer of sodium chloride into the cellular phase.

On the twelfth day of the study resins were discontinued. Thereafter, on days 14 and 15, 107 mEq. and then 80 mEq. of KCl were given by mouth. A delayed and relatively prolonged hyperkalemia was noted, with a transient rise in the serum sodium to a peak of 143 mEq. per L. (an increase of 27 mEq.). This rise was noted 25 hours after the last dose of KCl chloride. Concomitantly, serum chloride rose to a peak of 103 mEq. per L. The patient also reverted to regular sinus rhythm

TABLE II

External balance of electrolyte and maximal changes in serum sodium after KCl administration *

Pt.† Date	Maximal changes in serum Na	Time of maximal serum Na: After start	Time of maximal serum Na: After last dose	Duration of the concurrent balance study	External balances‡ for the entire period: Na	K	Cl	Body wgt.	Calculated mEq. shift§ intra and extracellular balances: Sodium ECW	Sodium ICW	Potassium ECW	Potassium ICW
	mEq./L.	*hours*		*hours*	*mEq.*	*mEq.*	*mEq.*	*Kg.*				
J. C. 3/7	+27	56	25	72	+23	+282	+212	57	+324	−301	+45	+237
J. C. 3/18	+ 9	26	2	48	+ 1.2	+101	+ 92		+108	−107	+ 6	+ 95
F. P. C. 4/9	+ 9	48	12	48	+27	+104	+206	86	+156	−129	+20	+ 84
F. P. C. 4/17	+ 9	26	1	48	+28	+101	+166		+156	−128	+20	+ 81
F. S.	+ 5	24	12	24	+ 4	+ 76	+ 92	56.5	+ 60	− 56	+12	+ 64
A. M. 1/6	+ 2.5		12	120	+ 0.2	+183	+116	58.1	+ 30	− 30	+15	+168
A. M. 1/20	+ 2.5	24	12	24	+14	+ 40	+ 45		+ 30	− 16	+ 9	+ 31
J. V.	+12	30	1					43.5	+117		+30	
W. McL.	+ 7	6	1					70	+ 98		+33	

* This table presents data indicating that large internal shifts of sodium ion have occurred after KCl which cannot be accounted for by change in overall sodium balance. Calculations of intra and extracellular balance are presented to depict the type and direction of internal change of electrolyte. An error in these estimated changes is the fact that maximal serum changes often occurred within nearest 24 hr. balance periods and often were subsiding at the time the nearest concurrent balance period collections were closed.

† The first two patients were each studied on two different occasions. All were on constant low sodium diets excepting Pt. A. M. who on the second study was given a constant but higher sodium intake.

‡ External balance = sum of intake minus output (urine and stool) for the stated period during which KCl was being administered. No corrections have been made for insensible loss.

§ Calculated balances are based on the assumption that ECW is about 20 per cent of body weight (see Text). The ECW balance is thus calculated as maximal increase in Na and K, respectively, times the assumed ECW volume. The ICW balance represents the difference between estimated ECW balance and observed external balance.

TABLE III

The effect of KCl administration on body weight and on the urinary excretion of sodium and water

Pt. Date	Body weight		24 hr. urine volume		24 hr. total urine sodium	
	Control*	After KCl	Control*	During KCl	Control*	During KCl
	Kg.		*cc.*		*mEq.*	
J. C. 3/7	56.9	57.4	780	580	0.9	1.3
J. C. 3/18	57.6	57.0	970	455	1.1	2.3
F. P. C. 4/9	86.4	86.7	1250	1485	0.2	0.9
F. S. 12/6	56.1	55.9	700	375	0.9	1.4
A. M. 1/6	58.1	57.6	1223	1145	16.9	17.0
A. M.† 1/26	57.3	57.8	1155	1050	32.3	39.4
J. V. 4/14	42.8	43.9	1275	975	—	—
W. Mc. 5/5	—	—	1600	1550	—	—

* Control is the average of 3 control day measurements. During KCl is the average daily excretion while on the drug. After KCl is the weight at the end of period of administration.

† A. M. was studied on a higher as well as low salt diet.

when the serum potassium reached 9.5 mEq. per L. and remained in sinus rhythm for several months thereafter.

The large increase in the extracellular concentration of sodium which was noted in this patient as well as in others could, theoretically at least, be attributed to any of the following factors:

1. A positive overall sodium balance;
2. A decrease in the extracellular fluid volume without a commensurate change in the total amount of extracellular sodium;
3. A migration of sodium from the intra to the extracellular fluid space.

No additional sodium was administered and only minor alterations from control periods occurred in sodium excretion when KCl was given (Table III). These small changes in sodium balance following KCl administration could not possibly account for the observed fluctuations in serum levels of sodium. For example, in patient J. C., the maximal rise in serum sodium concentration of 27 mEq. per L. occurred at a time when both sodium intake and excretion were not significantly altered (Tables I, II, and III).

Evidence pertaining to possible changes in extracellular fluid volume may be summarized as follows. At the time when the peak change in serum sodium was noted, there was no significant change in weight, or increase in urine volume. Furthermore, in all cases changes in serum protein and hematocrit were minimal and revealed no consistent evidence of hemoconcentration. Changes in extracellular fluid volume were calculated by the method of Darrow (18) and these also failed to reveal any contraction of extracellular fluid space. Some examples of these calculations are given below. The possibility that the serum sodium level increased because of a contraction in the extracellular space may, therefore, be dismissed from further consideration and changes will be described assuming no significant changes in extracellular volume. A shift of sodium from the intra to the extracellular compartments is, therefore, perhaps demonstrated in this and subsequent patients by exclusion of above factors.

Assuming patient J. C. to have an extracellular volume of at least 12 liters and that the increase in serum sodium concentration represented a trans-

TABLE IV

Metabolic balance data—Patient F. P. C.

Cirrhosis F. P. C. 62 ♂		Potassium *mEq. per 24 hr.*				Sodium *mEq. per 24 hr.*				Chloride in urine†	wgt.	Serum					
			Excretion				Excretion										
Period*	Day	Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance			K	Na	Cl	Prot.	Hematocrit	Time of day
										mEq./24 hr.	*Kg.*		*mEq./L.*		*Gm.*		
	1	72.8	27.0	12.2	+33.6	14.9	0.56	0.63	+13.7	8.0	85.8	4.5	126.0	101.7			8 a.m.
	2	72.8	30.0	12.2	+30.6	14.9	1.10	0.63	+13.2	7.5	85.7						8 a.m.
	3	72.8	43.0	12.2	+17.6	14.9	0.80	0.63	+13.5	9.5	85.5	5.0	126.0				8 a.m.
Control	4	72.8	46.6	12.2	+14.0	14.9	0.13	0.63	+14.1	6.7	85.4						8 a.m.
	5	72.8	52.0	19.3	+ 1.5	14.9	0.24	0.53	+14.1	8.0	85.3	4.6	125.4				8 a.m.
	6	72.8	46.0	19.3	+ 7.5	14.9	0.09	0.53	+14.3	5.9	86.3						8 a.m.
	7	72.8	55.0	19.3	− 1.5	14.9	0.20	0.53	+14.2	7.8	86.2	4.9	125.9	101.4		42.1	8 a.m.
	8	72.8	47.3	19.3	+ 6.2	14.9	0.34	0.53	+14.0	6.3	86.4	5.1	124.2		7.2	42.1	8 a.m.
10 Gm.	9	207.0	138.5	16.5	+52.5	14.9	0.80	0.35	+13.8	45.8	86.6	4.5	124.2	100.1	7.0	42.1	8 a.m.
KCl												6.7	124.4	105.6	7.1	40.0	7:30 p.m.
14 Gm.	10	260.4	192.2	16.0	+52.2	14.9	1.20	0.35	+13.4	109.0	86.8	5.4	126.2	108.0	7.2	40.1	8 a.m.
												7.25	131.0	111.0	6.8	38.0	3 p.m.
												7.5	130.0	111.4	7.4	39.9	8 p.m.
	11	72.8	120.5	16.0	−63.7	14.9	0.59	0.35	+14.0	69.7	86.7	5.4	133.0	110.2	7.4	41.5	8 a.m.
												5.4	131.0	109.6	6.6	38.0	2:30 p.m.
	12	72.8	71.2	16.0	−14.4	14.9	0.31	0.35	+14.2	33.0	86.4	5.3	128.4	106.4		38.9	9:30 a.m.
	13	72.8	58.4	16.4	− 2.0	14.9	0.26	0.49	+14.2	26.2	86.3		127.5	106.0			
Control	14	72.8	48.8	16.4	+ 7.6	14.9	0.14	0.49	+14.3	19.4	86.4	5.2	126.5	105.4			8 a.m.
	15	72.8	49.6	16.4	+ 6.8	14.9	0.16	0.49	+14.3	17.2	86.4	5.2	125.8	105.4			11:30 a.m.
	16	72.8	50.4	16.4	+ 6.0	14.9	0.15	0.49	+14.3	13.1	86.7	5.1	126.2	97.0	7.1		8 a.m.

* Urine collections were made every twenty-four hours at 8 a.m.
† Diet chloride constant at 19.3 mEq. daily.

fer of sodium into the extracellular space, this alteration would represent a mobilization of 324 mEq. of sodium. If this increase in extracellular sodium resulted from cellular transfer, then the question of whether it exchanged with potassium ion may be raised. Only 312 mEq. of potassium were administered, including that of the diet, and at the time of maximal change in serum sodium some 20 to 30 mEq. had been excreted. The peak serum level of 9.5 mEq. per L. might account for another 45 mEq. Some 237 mEq. of potassium, therefore, would be available to enter cells and exchange with sodium.

The administration of KCl was repeated in the same patient. When 107 mEq. of KCl were added to the diet the serum sodium rose by 9 mEq. per L., representing an apparent increase in total extracellular sodium of 108 mEq. At the time of maximal rise in sodium the serum potassium was increased by 0.5 mEq. per L. and, at this time, roughly 20 mEq. of potassium had been excreted. Including the diet, this could leave an excess of about 95 mEq. of potassium, either in cells or intestine, which was associated with the rise in serum sodium. By these estimations it appears again that the increase in extracellular sodium could perhaps be accounted for by an exchange with potassium. Serum protein concentration and hematocrit revealed no changes to suggest transfer of water from the extracellular fluid which might also have been a factor in the rise in extracellular sodium. Finally, this patient was given NH_4Cl in an amount equivalent to the KCl previously administered, and no changes in serum sodium and potassium were observed, suggesting that potassium ion is necessary for the production of the observed phenomenon.

Data for a second patient, F. P. C., with decompensated Laënnec's cirrhosis, are presented in Figure 1 and Tables I through IV. While on a constant low sodium diet KCl was administered (322 mEq. in two days) and an increase in the serum sodium concentration of 9 mEq. per L. was observed. This massively edematous man had an assumed extracellular volume of 17.3 liters. *Ceteris paribus,* the change in the serum sodium concentration would represent the mobilization of at least 156 mEq. of sodium into the extracellular space. This rise occurred at a time when more than 100 mEq. of administered potassium remained within the body, largely within cells. There was no significant change in serum protein concentration.

Balances between the intra and extracellular compartments were calculated by the method of Darrow (18) assuming an obligatory extracellular position for chloride and also assuming an extracellular fluid volume of 20 per cent of body weight at the start of administration of KCl. The serum sodium rose from 124 to 133 mEq. per L. during the 48-hour period of KCl administration. With an assumed initial extracellular space of 17.3 L. this space was calculated to be 17.6 L. at the end of the 48-hour period. On the basis of these values and the changes in serum sodium concentration, the calculated distribution of sodium was: external balance + 26 mEq.; extracellular balance + 190 mEq.; and intracellular balance − 164 mEq. The comparable values for potassium were: external balance + 104 mEq.; extracellular balance + 16 mEq.; and intracellular balance + 88 mEq. With this type of calculation, approximate estimates for the first patient, J. C. also indicate that the rise in serum sodium of 27 mEq. per L. represented the migration of about 300 mEq. of sodium from the intracellular space with a positive intracellular K balance of about 237 mEq. Within the limits of accuracy of these calculations, the data for all patients indicate that the rise in serum sodium can be attributed to a cellular exchange for potassium.

The KCl was administered over a twelve-day period in progressively increasing dosage to a maximum of 295 mEq. daily (patient F. P. C.). Similar changes in serum sodium were observed, again without related variation in serum protein. Of note is the recurrent tendency for the serum sodium to return to lower levels shortly after each increase in KCl dosage.

In Figure 1 and Tables I, II, and III data are presented from a similar study, performed without balances, on patient J. V. This patient was a 43-year-old woman with a two-year history of accelerated hypertensive disease. She had a serum protein concentration of 5.4 Gm. per cent. Her disease process was considered by some to represent a diffuse collagen disease. There was no detectable edema. On a moderately low sodium diet the serum sodium was found to be consistently in the 120 to 123 mEq. per L. range. There also was

observed a consistently elevated serum potassium in the range of 6.0 mEq. per L. She was given 240 mEq. of KCl orally in two days. The serum sodium concentration increased 12 mEq. per L. Calculating as previously, the change could represent an extracellular increment of at least 117 mEq. of sodium. The serum potassium reached a peak of 9.1 mEq. per L. No accurate estimate can be made of the amount of potassium within cells, but necessarily the balance is strongly positive and might approach 150 mEq. In this patient the rise in serum sodium concentration was more sustained. Serum proteins, in contrast to the first two subjects, increased slightly with the rise in serum sodium (5.4 to 5.6 Gm. per cent), suggesting the possibility of some degree of hemoconcentration and water shift into cells.

Similar observations were made on a febrile man with tuberculous meningitis (W. McL.). This patient's hyponatremia had previously been treated vigorously with sodium by mouth and hypertonic saline by vein without striking benefit. Control electrolyte values were more variable (Figure 1) but the change in serum sodium after 228 mEq. of KCl in one day appeared to be significant (+ 7 mEq. per L.) and, moreover, was relatively sustained. Serum proteins increased from 6.9 to 7.1 Gm. per cent, suggesting a slight reduction in extracellular fluid volume. This subject differs from the others in that considerably more potassium was given to produce a smaller rise in serum sodium concentration. Renal function was normal.

F. S. (Tables I, II, and III) was a 29-year-old man with Tetrology of Fallot and edema. Before treatment serum electrolyte values were: sodium 111 mEq. per L., potassium 4.0 mEq. per L., chloride 79 mEq. per L., CO_2 25 mm. per L. Following administration of 94 mEq. of KCl in one day the serum rose to 117 mEq. per L. without change in weight and, again, urine volume was reduced. Assuming an extracellular volume of only 12 liters, the rise in sodium could represent a mobilization of 60 to 70 mEq. of sodium into the extracellular fluid. At the time of the change in serum sodium this patient retained about 64 mEq. of potassium outside of the extracellular space. In contrast to all of the other edematous patients, the rise in serum sodium produced in this man was followed by a diuresis. Fifteen days later serum sodium was 130 mEq. per L. and three days thereafter 140 mEq. per L.

Subject A. M., Tables I, II, and III, was a 47-year-old patient with a more normal serum sodium concentration. This woman, with cor pulmonale, was massively edematous and her serum sodium concentrations varied from 135 to 138 mEq. per L. While on a low sodium diet she was given relatively small, gradually increasing doses of KCl over a five-day period; the total dose given was 563 mEq. of KCl. A diuresis was not induced. The serum sodium rose to 140 mEq. per L.—a small change perhaps not exceeding the error of the method. After a control period on a higher sodium intake (67.5 mEq. daily) KCl was given again. Even with this higher sodium intake no diuresis was observed after potassium administration, nor did the diet with its higher salt content affect the serum sodium. There was, however, another slight increase in serum sodium from 137 mEq. per L. to 140 mEq. per L. when potassium chloride was added.

The changes in serum chloride concentration in these patients, in general paralleled the changes in serum sodium. The CO_2 content of the blood showed a general tendency to fall slightly during KCl administration.

The three hyponatremic edematous subjects were all excreting less than 1 mEq. of sodium a day while on constant low sodium diets (Table III). When KCl was given to these patients there was no significant change in sodium excretion despite simultaneous increase in the serum sodium concentration. Moreover, potassium did not produce significant changes in sodium excretion in an edematous cardiac patient (A. M.) who had a higher serum sodium level. This was also true after dietary sodium had been increased.

The twenty-four hour exchangeable sodium (Na^{24}) mass was measured in the first two patients before and after KCl. In patient J. C., the exchangeable sodium which was measured on two occasions before and again twice after KCl revealed an increase of 300 mEq. in the exchangeable sodium after KCl. These data are suggestive also of an increase in sodium concentration as a result of an increase in the total exchangeable pool secondary to mobilization of previously "bound" sodium by exchange with potassium. In contrast,

however, in patient F. P. C. no significant change was observed.

DISCUSSION

The present study illustrates that in hyponatremic patients, a significant rise in serum sodium concentration may follow the oral administration of potassium chloride. In the absence of a significant change in overall sodium balance the observed phenomenon must represent either (a) a loss of water from the extracellular fluid, or (b) a release of sodium into the extracellular fluid, or (c) a combination of the two.

With reference to the first possibility, no definite or consistent evidence of hemoconcentration was obtained. However, small but significant fluid shifts may have been masked by the known diurnal and postural variations in serum protein concentrations (19). When the distribution between intra- and extracellular phases is calculated by Darrow's method, no significant changes in the chloride space were observed. The positive balance of chloride was associated with a rise in chloride concentration. In addition, if water moved from the extracellular fluid to the cells along with potassium, this should have produced increased osmolarity of the extracellular space which of necessity, would give rise to osmotic inequality. Thus, if the osmotic pressure is raised outside of cells, it ought to be correspondingly increased inside.

With reference to the second possibility, *i.e.*, the extracellular increment of sodium by redistribution from cells or other depots, only circumstantial evidence has been obtained, by exclusion of other likely possibilities. It has been established that under various circumstances sodium may replace potassium in cells. Thus, sodium can replace intracellular potassium after administration of desoxycorticosterone (20), in rats raised on a low potassium diet (21), in hypokalemic alkalosis (22), and in infantile diarrhea (18). Moreover, other investigators (23), studying familial periodic paralysis, have obtained evidence in one case of reciprocal sodium and potassium changes. That a sodium reservoir exists is suggested by the more recent work of Bergstrom (24) in rats, which indicates that, within 48 hours following base depletion, bone can release considerable sodium and potassium. In addition, Levitt, Turner, and Sweet (25) report that, during venous obstruction in man, a rise in the inulin space may be accompanied by an increase in extracellular sodium chloride. These analogies also make the supposition that potassium has exchanged with sodium the more likely explanation of the results observed in the present study.

Abnormalities of the intracellular fluid in the disease states under study have be demonstrated. Analyses of biopsy specimens of skeletal muscle of hyponatremic patients in congestive heart failure suggest that there may be more sodium and less potassium than normal within cells (3, 26). However, the reported data are not altogether consistent (27). Other investigators (1, 28–30) have reported retention of potassium in excess of nitrogen in patients recovering from congestive heart failure. Elkinton, Squires, and Bluemle (31), have concluded on the basis of their data that in cardiac failure intracellular abnormalities consist of potassium depletion, with associated increase of osmolarity of solutes present in the cells, and cellular overhydration. Fox, Friedberg, and White (32) had previously reported that the administration of potassium and sodium salts to patients in heart failure resulted in a positive potassium balance. These data suggest, but do not prove, that in heart failure sodium may pass through the cell membrane and potassium may leave the cell. Moreover, in experimental hypertension (33, 34) and in human hypertensive disease (35) evidence has been reported suggesting increased intracellular sodium content. In tuberculous meningitis, Harrison, Finberg, and Fleishman (36) found an increased sodium and decreased potassium content of muscle.

The data presented here afford evidence that, in the patients of this study, a large fraction of the administered potassium is stored, at least temporarily, beyond the confines of the extracellular fluid. These observations are in agreement with previously reported data. According to the studies of Winkler, Hoff, and Smith (37), the increase in concentration of serum potassium after an ingested or injected dose is only one-third as high as would be expected were its distribution purely extracellular—about two-thirds of the absorbed potassium leaves the extracellular fluid. Other investigators (38) have subsequently administered K^{42} to animals and shown that it is rapidly taken

up, first by the viscera, probably with an anion, and then slowly released to muscles and other tissues (50 to 60 per cent of body potassium exchanges in 12 hours). The tissues of the body therefore seem capable of storing, at least temporarily, large amounts of potassium; *i.e.*, as Winkler has stated the body has a buffer capacity with respect to potassium. The transport of potassium into cells is conditioned evidently by metabolic activity (39); when it enters cells osmotic equality may be maintained by entrance of water, by exit of sodium, by change in activity of cell cations and anions.

In contrast to the large amounts of potassium ion tolerated by normal subjects (9, 10, 40) and even by patients with renal insufficiency (37), the patients of this study developed significant hyperkalemia with relatively small doses of potassium chloride and in some the return to normal serum potassium levels appeared to be delayed (Table I). Brown, Tanner, and Hecht (40) have reported on similar observations in a group of cardiac patients, and point out that this finding is of particular interest since it has been stated that it is difficult, if not impossible, to produce potassium poisoning except in patients with renal failure, shock, or adrenal insufficiency (22). Indeed, Winkler, Hoff and Smith (37) were unable to produce fatal potassium poisoning in normal dogs unless they ligated the ureters.

The diminished capacity of edematous (41) and hyponatremic subjects to excrete electrolyte may be in part responsible for the magnitude of the observed phenomena. When KCl is given, this represents the addition to the body of primarily obligatory extracellular anions with a largely intracellular cation. The observed rise in serum sodium could be attributed to decreased capacity to excrete potassium itself, and then subsequent to exchange of this potassium for intracellular sodium, the continued inability to excrete sodium, chloride, or bicarbonate. The fall in bicarbonate in all of these patients after addition of large amounts of chloride was always small and wholly inadequate to compensate for the chloride increase.

The sensitivity to potassium of the group reported here could, therefore, be the result of a renal insufficiency. However, only one of the patients had a primary renal disease and, moreover, patients with advanced renal disease often show no elevation of serum potassium even when the intake of potassium is high. This sensitivity, as a second possibility, may represent deficient capacity of body tissues to store potassium, and may also be specifically related to the concentration of, or absolute quantity of sodium in the body. This would perhaps be analogous to the tendency to hyperkalemia and potassium sensitivity observed in adrenal insufficiency. A preliminary injection of NaCl in animals will protect them from lethal doses of potassium (42), but this may be the result of specific cardiac protection.

The failure of potassium chloride to augment water or sodium excretion in the edematous patients studied occurred despite the fact that the low serum sodium levels often rose strikingly towards normal, suggesting perhaps that the concentration of serum sodium is not *per se* an important factor in sustaining sodium retention. This failure of diuresis of edematous hyponatremic subjects is in contrast to the response of normals and certain edematous states as reported by others.

SUMMARY

Potassium chloride was administered orally to hyponatremic patients with and without edema.

In these patients small to rather striking increases in the serum sodium concentration were observed without the addition of exogenous sodium.

The nature of the phenomenon is not wholly clarified but seems best explained by postulating a release of sodium from cells in exchange for potassium.

The estimated release of sodium ion was of the order of 60 to 300 mEq. The actual change was probably in excess of these values since the extracellular volume was estimated to be only 20 per cent of the body weight in patients often massively edematous.

In the edematous hyponatremic subject no significant change in renal excretion of sodium or water was observed, after potassium chloride administration, even when serum sodium levels were simultaneously raised towards normal. This occurred with both low and higher sodium intakes.

The hyponatremic patients studied seemed unable to metabolize potassium with normal facility and its administration is therefore not without

hazard; when compared to normal subjects the hyperkalemia produced seemed to be more profound and more prolonged. Moreover, with the doses employed in this study potassium chloride did not exert a diuretic action.

ACKNOWLEDGMENT

The author is indebted to Dr. Gilbert H. Mudge and to Dr. George A. Perera for advice and assistance. He is also indebted to Misses Margaret Crymble and Ann Richards and Mrs. Laura Losin for technical assistance.

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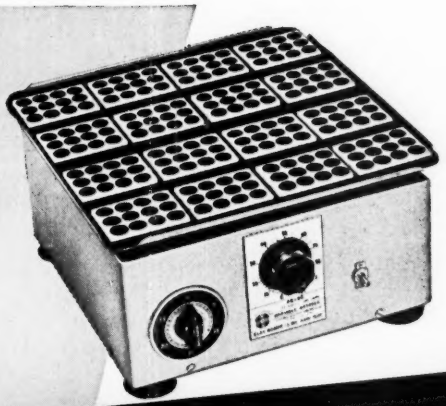

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INTRODUCTION

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An additional use has been recently reported for hyaluronidase as a topical aid to surgery in the separation of adherent viscera. As in other uses for the enzyme, the rationale is provided by the lytic effect on hyaluronic acid.

INDICATIONS

The topical application of hyaluronidase solution is regarded by the author[1] as a means of considerably facilitating the separation of adherent vital organs. Special value is suggested in the portal triad, lung hilum, or retrothyroid region, where dissection in the presence of residual fibrosis may be hazardous.

Hyaluronidase seems to have no effect on intact pleural or peritoneal surfaces. Its use is contraindicated in active infection or malignancy at the operative site.

EXPERIMENTAL STUDIES

To evaluate the effects of hyaluronidase in dissection, canine surgery was performed in various anatomical regions. In the portal triad, separation of the portal vein, common duct, and hepatic artery was greatly simplified by the application of hyaluronidase solution. After exposure of the lung hilum, similar ease was experienced in separating the pulmonary vessels and bronchi from the lung tissue. In the neck, dissection of the blood vessels, vagus nerve, thyroid gland, trachea, and esophagus was also facilitated by hyaluronidase application. No deleterious effects were noted.

CLINICAL SURGERY

Favorable results were recorded for all but three of twenty-seven operations, performed by ten different surgeons. The procedures included submucous resection, thyroidectomy, pneumonectomy, pulmonary lobectomy and decortication, subtotal gastrectomy, cholecystectomy, common-duct exploration, inguinal herniorrhaphy, bowel obstruction from multiple adhesions, sympathectomy, and ligation of the popliteal vein. No undesirable effects were observed. As for wound-healing, the postoperative course was uneventful in all patients, except for disruption of the bronchial stump in one case ten days after pneumonectomy.

METHOD

The hyaluronidase solution was prepared by dissolving eight vials of hyaluronidase (150 turbidity-reducing units each) in 5 cc. of sterile distilled water, providing a solution containing 240 TRU per cubic centimeter. It was found that warming this prepared solution to body temperature apparently potentiates the enzymatic effect. Cotton dissecting sponges were used for applying the solution to the adhesions. The maximal effect appeared in from two to four minutes.

REFERENCE

1. Streuter, M. A.: Surgery **34**: 28 (July) 1953.

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